Effects of selection for rapid development and early reproduction on male-female co-evolution and conflict in *Drosophila melanogaster*

A Thesis

Submitted for the Degree of

Doctor of Philosophy

By

Avani Mital

Evolutionary and Integrative Biology Unit

Jawaharlal Nehru Centre for Advanced Scientific Research

Bengaluru 560064, India

September 2019

Dedicated to my family

CONTENTS

DECLARATION

I declare that the matter presented in my thesis entitled "**Effects of selection for rapid development on male-female co-evolution and conflict in *Drosophila melanogaster***" is the result of studies carried out by me in the Evolutionary Biology Laboratory, Evolutionary and Integrative Biology Unit of Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, India, under the supervision of Prof. Amitabh Joshi, and that this work has not been submitted elsewhere for any other degree.

In keeping with the general practice of reporting scientific observations, due acknowledgement has been made wherever the work described has been based on the findings of other investigators. Any omission, which might have occurred by oversight or error of judgment, is regretted.

Avani Mital

Place: Bengaluru

Date: 18th September 2019

Evolutionary and Integrative Biology Unit
Jawaharlal Nehru Centre for Advanced Scientific Research
Bengaluru 560064
India

CERTIFICATE

This is to certify that the work described in the thesis entitled "**Effects of selection for rapid development on male-female co-evolution and conflict in *Drosophila melanogaster***" is the result of investigations carried out by Ms. Avani Mital in the Evolutionary Biology Laboratory, Evolutionary and Integrative Biology Unit of Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, India, under my supervision, and that the results presented in this thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

Amitabh Joshi

(Professor)

Place: Bengaluru

Date: 18th September 2019

Acknowledgements

First and foremost, I would like to thank my family for providing me the immense emotional support that I needed during the tenure of my doctoral research. Without their constant encouragement it would have indeed been very difficult to reach this stage. I am deeply grateful to my parents for being understanding, for teaching me humility through their actions, and for their unwavering faith in my abilities. I thank my brother, Amod, and my closest cousins, Smriti, Chirag, Samrit, Zoya, Vaidant and Aarushi for being the most wonderful family and friends. Their unconditional love has always been a pillar of strength. I want to thank my aunts and my uncle, they have been nothing less than parents to me and I am grateful to them for their positive guidance throughout my life. Finally, I want to thank my grandparents for always encouraging me to study hard and keep learning. They have been far ahead of their times and I greatly appreciate the values that they have raised me to hold.

Second, I want to thank Chakri, who has been my partner through good and difficult times and has had the unnerving talent for pointing out exactly what is going wrong with me. I cannot express in words how thankful I feel to have found a partner in him. He has, in a very direct way, made me a much better person and, consequently, a much better scientist. Whatever has changed for the better about me over the past two years has been a direct result of his company, friendship and love.

I also want to thank Dr. Elizabeth Daniel, who has helped me in the most crucial way through the course of this dissertation work. I am proud to say that I submit this thesis knowing that I have crossed personal hurdles and achieved great personal growth. Dr. Elizabeth showed me the way and I will always be grateful that I got a chance to meet her and gain her friendship.

I am grateful to all my batch-mates, Manaswini, Shveta, Lakshmi, Sunaina, Surabhi and Vikas , for keeping this journey fun and relaxed these past eight years. I would also like to thank the many friends I have made along the way: Rafi, Vybhav, Praveen, Croor, Shaurya, Asutosh, Soumyadeep, Kamini, Zenia, Srilakshmi and Aishwarya. Their company and friendship has made this journey all the more enjoyable. If I have missed a few friends, I can only say that their contribution has been significant and sooner or later I will remember those names and regret not having mentioned them here.

Srikant, Sajith, Neha, Dr. Joy Bose, Dr. Bodhisatta Nandy, Manan, Satya, Anjali and the late Dr. Snigdhadip Dey have been excellent lab-mates and great company. Srikant, Sajith, Manaswini, Neha and Dr. Joy Bose, especially, made my time in the lab memorable and I will miss working with them. There are many summer students and short-term students I would like to thank for their hard work and the light-hearted fun they brought to the lab atmosphere. Ratna and Pritha, especially, helped me with major experiments reported in this thesis.

I would also like to mention Dr. T. N. C. Vidya. Her teaching, guidance during a short-term project, constant support and encouragement helped me wade through some difficult times. Her friendly nature made it easy for me to approach her and she has been an excellent advisor to me for the last eight years. Dr. Sinha first motivated me to pursue scientific research and my admission to JNCASR was a direct consequence of his encouragement. Dr. Sheeba Vasu and the late Prof V. K. Sharma have been great teachers and I learnt a lot during the short-term projects I did under their supervision. I am grateful to them all.

Finally, and most importantly, I would like to express my gratitude to Prof. Amitabh Joshi. He has been a phenomenal teacher, both inside and outside the class room. There are practical lessons of life I have learnt from him and would like to state that not only has he given

me the opportunity to explore this subject in my own time and in my own way, but has also provided me both professional and personal support throughout my PhD tenure. He has truly been a mentor and I am thankful to him for the confidence he has had in my abilities. I would consider myself a successful scholar of science if I manage to teach even half as well as he does. There is a lot that I owe to his guidance and, needless to say, this thesis would not have been completed without his contributions as my teacher and supervisor.

Summary

The process of development is a crucial stage in the life-history of any organism, forming the bridge between hereditary factors that are transmitted and the phenotype that is exposed to selection pressures. Development time is, therefore, under strong selection for optimization to produce the most fit phenotype given the genotype and its environment.

Theoretically, short development time should be under strong positive selection. However, many tradeoffs have been revealed via experimental evolutionary studies on *Drosophila melanogaster*, showing decrease in adult fitness, both in terms of survival and reproduction, as a consequence of selection for rapid development. Through these and other experimental evolution studies on various life-history related traits, laboratory selection using *D. melanogaster* has proved to be an excellent tool for revealing genetic and phenotypic correlations that limit trait evolution, especially those relating to developmental duration.

In this thesis, I report studies on a set of replicate *D. melanogaster* populations that have been under long-term selection for rapid development and early reproduction, relative to ancestral controls, and specifically explore the relationship between rapid development and reproduction related traits in affecting sexual selection and sexual conflict experienced by adult flies.

Sexual selection was predicted by Darwin to be a significant form of selection that could influence evolutionary divergence via reproductive isolation, leading to speciation. Over the last 40-50 years, the theory of sexual selection has been expanded, defining various features of sexually reproducing, dimorphic animal populations, such as sex roles and various forms of sexual antagonism, that directly affect the evolution of traits in the two sexes, leading to co-

evolutionary arms-races between them. This phenomenon of sexual conflict has since become an important focus of research for understanding speciation.

The populations used in the following work have earlier shown incipient reproductive isolation from their ancestral controls, the first step toward speciation. These results prompted an exploration of whether sexually antagonistic selection could be mediating this divergence. The investigations reported here were aimed at understanding the effects of selection for rapid development, selection for early reproduction, and their interaction on the evolution of inter-locus sexual conflict in these populations.

I first discuss in detail our current understanding of sexual selection, sexual conflict and life-history evolution based on studies using *Drosophila sp*., and introduce the study system in detail (Chapter 1). Many traits that have traditionally been considered evidence for inter-locus sexual conflict in fruit flies have diverged between our selected and control populations. Preliminary investigations demonstrated the role played by faster development, and consequently a smaller adult body size, in affecting sexual conflict. However, differences in their reproductive behaviour, most likely a result of selection for early reproduction, are also large enough to impose different levels of direct, but unintended sexual selection in these populations (Chapter 2). This prompted a new line of investigation with these populations as different levels of sexual selection can directly lead to different levels of sexual conflict. I have used relaxed or reversed selection to understand the contribution of the two selection pressures, i.e., that for rapid development and for early reproduction, in mediating the changes in sexual conflict reported in these populations so far (Chapter 3). I discuss three possible hypotheses that explain these results: a) that a body size based trade-off adequately explains the decrease in sexual conflict, b) that early reproduction results in breeding conditions for lower sexual conflict, and c) that early

reproduction can change the nature of sexual conflict in the selected populations as compared to their ancestral controls, causing traits typically under conflict to no longer be under conflict. My results indicate strong support only for the first hypothesis (Chapters 4 and 5). Further, I propose that the strength of this first selection pressure (that for rapid development) is strong enough to affect adult reproductive behaviour, thus creating conditions for lower sexual selection as well. I conclude that although incipient reproductive isolation has been brought about through traits typically under sexual conflict in fruit flies, changes in those traits in this system of laboratory populations are a direct consequence of selection for rapid development, an important life-history related trait, rather than a direct consequence of the unintended changes in sexual selection (Chapter 6). Finally, I propose potential future work that will enable us to better understand the system (Chapter 6).

Chapter 1:

Introduction

The process of development is central to the realization of the Darwinian fitness of any organism, as it is in this stage of the life of an organism that hereditary factors subjected to selection in the previous generations give rise to the phenotype that is 'visible' to natural selection in the current generation. Theory suggests that the ideal life-history would involve almost instantaneous development and infinitely long survival, with a high rate of reproduction throughout (i.e. absence of ageing) (Roff 1992). However, any organism has finite resources and time at its disposal, and the allocation pattern of the same towards survival or reproduction, reflected in its life-history, is expected to be optimized for its ecology, through the process of selection. As ideal life-histories do not exist, these constraints that limit the simultaneous maximization of all fitness related traits remain an important focus of research in the study of biological evolution.

In sexually reproducing organisms, resource allocation to reproduction is further complicated by the need to find and successfully interact with individuals of the opposite sex, both of which can also affect the survival probability of an individual, imposing additional fitness costs of successful reproduction on the organism. This necessary interaction with individuals of the opposite sex becomes an important selection pressure (sexual selection), leading to inter-sexual co-evolution that also contributes to shaping the life-history of organisms.

Laboratory selection experiments, particularly with *Drosophila melanogaster*, have been very successful in enhancing our understanding of trade-offs among fitness related traits, with over 30 years of work (reviewed in Prasad and Joshi 2003). *D. melanogaster* has been especially amenable to such studies because its short generation time, and easy and cheap maintenance, allow long term, laboratory selection experiments with proper population level replication, wherein manipulation of specific life-history related traits can yield evolutionary changes that

reflect the constraints mentioned above. The long history of study of *D. melanogaster* in the laboratory adds to these benefits by providing information on the basic biology and laboratory ecology of the species allowing a good appreciation of selection pressures experienced by the study populations in the immediate past, which, in turn, helps us understand many nuances of the adaptive evolutionary process, including the lability of genetic correlations among fitness related traits (Harshman and Hoffman 2000, Archer et al 2003, Joshi et al 2003, Phelan et al 2003). In this thesis, I record investigations made into the male-female co-evolutionary processes in such long-term laboratory populations of *D. melanogaster*, wherein the rate of development has been the focal point of selection.

Constraints on Development Time

D. melanogaster is a holometabolous insect and, therefore, acquires most of its life-time food resource and final size during its developmental stages (Bakker 1959). As an adult, opportunities to significantly improve resources (usually reflected in adult body size) are limited and basic allocation patterns for various survivorship related and reproduction related traits may already be fixed at eclosion (Bakker 1959, Djawdan et al 1996). The adult size/weight of the individual is a reasonable indicator of its resource stores, and strongly determines many adult fitness related traits, both in terms of reproduction and lifespan (see Prasad and Joshi 2003 for a review). Therefore, any alteration of the developmental process or duration that affects adult body size in *D. melanogaster* is likely to have direct consequences on the fitness of the individual.

This thesis reports studies on a set of *D. melanogaster* populations that have undergone selection for rapid development and early reproduction, relative to controls, in the laboratory for

more than 600 generations (first described in Prasad et al 2000). These are the Faster developing, Early reproducing JB (FEJ) populations, the Joshi Baseline (JB) populations being their ancestral controls that were not consciously selected for either rapid development or early reproduction. The direct response to this selection has resulted in the FEJ flies developing about 60 hours faster than control flies (Prasad 2004, Ghosh-Modak 2009). A number of trade-offs have been recorded as a result of speeding up development in these populations, with reductions observed in pre-adult survivorship (Prasad et al 2000, Prasad et al 2001), larval feeding rate (Prasad et al 2001), average pre-adult rates of weight gain and body size at eclosion (Prasad et al 2000), larval competitive ability (Shakarad et al 2005), adult pathogen resistance (Modak et al 2009), fecundity, longevity, and starvation resistance (Prasad 2004). Many other studies on *D. melanogaster* populations have demonstrated similar trade-offs with development time, either by directly selecting for faster development (Zwaan et al 1995a, Nunney 1996, Chippindale et al 1997a) or because of selection for other traits (Chippindale et al 1994, Chippindale et al 1998). This body of work has helped us understand the lack of rapidly developing organisms in the wild, even though theory predicts higher fitness of organisms with short compared to long development times, all else being equal (Roff 1992).

Most of these trade-offs with developmental rate in *D. melanogaster* are presumed to be a consequence of body size reduction, which is often positively correlated with development time (Zwaan et al 1995a, Nunney 1996). Although this result may not be consistent across selection regimes, such as selection for age at reproduction (Chippindale et al 1994), the amount of resource acquired during the larval stages has been shown to affect adult fitness in *D. melanogaster* (Djawdan et al 1996). In populations of *D. melanogaster* selected for adult starvation or desiccation resistance, higher larval growth rates as well as an increase in lipid and

glycogen assimilation rates, respectively, were seen (Chippindale et al 1996, 1998). Furthermore, any decrease in development time that is brought about by a reduced third instar duration is likely to result in smaller adult body size as the post-critical developmental duration, which is primarily responsible for increase in larval (and consequently pupal and adult) body size, is reduced. In the FEJ, the third instar larval duration has shown the maximum percentage reduction compared to the JB, significantly contributing to the 60 hour development time reduction (Prasad 2004).

The consequences of rapid development and the resultant resource reduction are also significant as sexual selection in *D. melanogaster* is known to operate on adult body size; large body size is often preferred by the opposite sex. Large males are usually better at winning mating opportunities (Dow and von Schilcher 1975, Partridge and Farquhar 1983, Pitnick 1991), either via direct contest competition or by being more attractive to females (Partridge et al 1987a, b). Large or more fecund females are also attractive to males (Bateman 1948, Pitnick 1991, Byrne and Rice 2006, Long et al 2009, Nandy et al 2012). In *D. melanogaster*, large body size has been correlated with greater reproductive success of both females and males (Mueller 1985, Zwaan et al 1995b, Bangham et al 2002, Houle and Rowe 2002). It is worth pointing out, however, that many of these studies rely on large differences in adult body size between the individuals being observed, a degree of body size variation not normally encountered within a laboratory population. However, at least between the FEJ and JB, the body size differences are quite large, FEJ flies being about half the size of JB flies at eclosion.

Sexual Conflict and Sexual Selection

An increase in body size in *D. melanogaster* is likely to affect the fitness of each sex differently due to differences in the strategies adopted by males and females, as discussed below. Inherent differences in parental investment made by the two sexes due to anisogamy (Bateman 1948, Trivers 1972, Parker 1979) often cause males and females to adopt different strategies, or sex roles (Darwin 1871, Bateman 1948), in order to optimize reproduction to maximize their respective fitnesses, within constraints. Anisogamy results in competition among the more abundant gametes (sperm) for the rarer gametes (eggs). Traditional sex roles are hence defined as follows: males tend to be less discerning, seeking as many instances of mating as possible, whereas females mate less and are more selective in their mate choice (Bonduriansky 2008, Schaerer et al 2012).

This difference in mating strategies typically results in a greater variance in male than female reproductive success (Andersson 1994). Essentially, males tend to benefit more from each additional mating than do females, and, therefore, the optimum mating rate for males is usually higher than that for females. Consequently, selection operates on mating propensity and related traits in opposite directions, depending on the sex of the individual (Chippindale et al 2001, Chapman et al 2003, Bonduriansky and Chenoweth 2009). This results in intra-locus sexual conflict that can often be resolved by resolved by sex-limited expression of genes (Rice 1996), leading to sexual dimorphism (Rhen 2000). The difference in mating strategies can also lead to inter-locus sexual conflict, which arises when one sex expresses a trait that improves its fitness, via interaction with the mating partner, often imposing a fitness cost on the other sex, which in turn may evolve counter measures, leading to a co-evolutionary arms race (Parker

1979). In particular, such sexual conflict involves males manipulating the endocrine-reproductive system of females in a manner that affects female fitness negatively, while increasing male fitness, resulting in sexually antagonistic co-evolution (Chapman et al 2003). Females are, in turn, under selection to evolve resistance or tolerance to such male manipulative traits (Chapman et al 2003.). In *D. melanogaster*, sexually antagonistic co-evolution is well studied and is triggered by males that physiologically and behaviourally manipulate females via chemicals produced in their accessory glands, known as SFPs (Seminal Fluid Proteins) or Acps (Accessory Gland Proteins) (Chapman and Partridge 1995, Chapman et al 2003, Rice and Holland 1997). These proteins are known to increase female egg output soon after mating (Chapman 2001, Wolfner 2009), and also to reduce female receptivity to re-mating (Chen et al 1988), among other responses, both of which are calculated to increase the proportion of progeny sired by that male. These chemicals collaterally also decrease female longevity (Fowler and Partridge 1989) and impact immune responses (Fedorka et al 2007, Imroze and Prasad 2011), thus imposing a direct cost on her fitness.

Whenever the fitness benefits of such manipulations outweigh the fitness costs incurred by reducing female fitness, they get selected, thus triggering the 'arms race'. The fact that preferred *D. melanogaster* males are more detrimental to female fitness (Pitnick and Garcia-Gonzales 2002, Friberg and Arnqvist 2003, Long et al 2009, Imroze and Prasad 2011), and are usually larger in size, indicates that in this species, the cost incurred by reduced female life-span is cancelled out by the benefits incurred by the male through manipulation. It is also likely that the intensity of selection on male manipulative traits will depend on the operational sex ratio (Emlen and Oring 1977).

In some species, changes in operational sex ratio or changes in investment patterns toward offspring have been shown to result in sex role reversal (Vincent et al 1992, Jones et al 2000). Since manipulation of females would probably be necessary for a male to maximize its reproductive fitness only under highly competitive mating conditions, the lab ecology of *D. melanogaster* has been manipulated in the lab (enforcement of random monogamy or sex-ratio manipulations) in order to investigate if lower levels of conflict might evolve with reduced male-male competition for females (Wigby and Chapman 2004, Linklater et al 2007, Edwards et al 2010, Nandy et al 2013 a, b). In promiscuous species such as *D. melanogaster*, competition can be acute after mating as well, requiring additional resources (Acps) for tackling sperm competition (Chapman et al 2003). Such post-mating effort rises particularly when females mate multiply, resulting in selection for compounds that aid in sperm storage and competition (Anrqvist and Nilsson 2000), and has been extensively studied in *Drosophila sp*. Moreover, shorter time available for breeding can also weaken the level of post-mating competition experienced by males, especially if there are fewer opportunities for females to re-mate. There is some evidence to suggest that extending the available breeding time does increase the male age-specific sperm offense and defense ability and their ability to induce typical female post mating responses (PMR) (Service 1993, Service and Fales 1994, Service and Vossbrink 1996). Since the FEJ populations are selected for early reproduction compared to JB populations, this can potentially create conditions for different degrees of male-male competition for mating with females, with FEJ females possibly having far fewer re-mating opportunities than do JB females.

Evolutionary biologists have recognized the necessity of understanding the relationship between intra- and inter-locus sexual conflict, sexual selection and life-history related traits such as time of reproduction and aging rate (Bonduriansky et al 2008, Wedell et al 2006), as well as

ecological factors relating to the same, such as nutrition (Amitin and Pitnick 2007, Fricke et al 2010, Edwards and Chapman 2012, Zikovitz and Agrawal 2013, Mason et al 2016, Schultzhaus and Carney 2017) and sex ratios (reviewed in Edwards and Chapman 2010, Nandy et al 2013 a, b, 2014, Wensing et al 2017). It is this multifaceted relationship that forms the basic framework within which this thesis work has been conducted, i.e. understanding male-female co-evolution and sexual selection within the ambit of life-history selection.

How selection on life-history related traits is affected by sexual selection is crucial to our understanding of evolution in sexually reproducing organisms. It is often presumed that life-history related traits will act to improve both male and female fitness uniformly across sexes, whereas sexual selection and sexual conflict will operate in a sex specific manner, constraining the evolution of the optimum life-history, and vice-versa (Bonduriansky et al 2008). For example, development time shows sexual dimorphism in insects, is a crucial life-history related trait and as discussed above, can affect adult fitness in terms of both survival and reproduction (Bonduriansky et al 2008, Bonduriansky and Chenoweth 2009). Mate choice by either sex can constrain or aid the evolution of traits such as increased body size, a trait that is likely to provide a fitness advantage to both males and females, all else being equal (Long et al 2009). Therefore, for a more complete understanding of trait evolution, detailed investigation of the interactions between natural and sexual selection pressures is essential.

Finally, sexual selection is believed to be an important factor bringing about reproductive isolation between populations that may ultimately lead to speciation. Intuitively, it makes sense that reproduction related traits would be fundamental in bringing about speciation. More recently, inter-locus sexual conflict has also been implicated in this process (Ritchie 2007), with adult ecology being the primary force behind it (Parker and Partridge 1998, Anrqvist and Rowe

2013, Ali et al 2017). However, the effect of the interactions of life-history selection and sexual selection on reproductive isolation is not very well understood (Maan and Seehausen 2011). The large divergence between the FEJ and JB populations in terms of so many important life-history traits begged the investigation into possible reproductive isolation, which directly led to the results that motivated this study.

Motivation for the Study

While setting up crosses between JB and FEJ populations for a quantitative genetics study, high mortality rate of FEJ females crossed with JB males was observed, indicating a possible decrease in inter-locus sexual conflict levels in the FEJ populations (Ghosh and Joshi 2012). Higher post-mating mortality of FEJ females when mated to control population males, compared to FEJ males, could be due to low resistance of FEJ females to mate manipulative proteins transferred by JB males, thus eliciting a more severe PMR than mating with FEJ males would have. Given that the FEJ females are also smaller in size than their JB controls, I created body size controls to the FEJ flies from the JB flies by limiting food availability during development to investigate the possibility of size dependent susceptibility to mate harm (Mital 2013). Smaller JB males, similar in size to FEJ males, were found to induce a weaker PMR in terms of female longevity, while smaller females from the JB genetic background retained resistance to the same. Co-evolution with FEJ males that had low levels of mate manipulative traits was suggested as a plausible explanation for loss of resistance in the FEJ females (Mital 2013).

However, since FEJ populations are selected for both rapid development and early reproduction, relative to controls, it was not clear if rapid development, and the consequent body

size reduction, were primarily responsible for the reduced level of inter-locus sexual conflict in the FEJ populations. At the very least, the shorter duration of time available for breeding in the FEJ populations could also reduce the competition experienced by males, potentially selecting for lower levels of mate manipulation. Moreover, the additional cost of mate manipulation is also expected to burden the already limited resources of the small FEJ flies. Thus, both selection pressures could have potentially given rise to lower inter-locus sexual conflict in the FEJ populations, explaining the results found by Ghosh and Joshi (2012).

It is worth noting that the role of reduced body size, shorter development time and inbreeding have been invoked many times in the past to explain reduction in male competitive ability (see Wigby and Chapman 2004, but countered by Rice and Holland 2005). I did not actively test for the role of inbreeding in the following work, focusing only on development time selection (and therefore a smaller body size) and early reproduction selection. However, my approach does enable me to address the possibility of inbreeding giving rise to these traits, if only suggestively, and this is discussed in the final chapter.

A detailed investigation of the contribution of the two selection pressures in lowering the inter-locus sexual conflict levels in FEJ populations is what is primarily explored here. First, the differences in their breeding ecologies are established, using a behavioural study approach. Second, reverse selection is used to tease apart the contributions of selection for rapid development and early reproduction, respectively, to lowering the level of inter-locus sexual conflict in the FEJ populations.

Reverse (Relaxed) Selection

In the context of laboratory experimental evolution, reverse selection refers to the "reimposition of the same selective pressure on derived populations as those of the recent ancestor populations" (Teotonio and Rose 2001). Reverse selection is one way of testing the effects of past history of selection on the robustness of trade-offs as well as investigating possible reduction in additive genetic variation for these traits (Joshi et al 2003, Phelan et al 2003, Archer et al 2003, Teotonio and Rose 2001). The reverse selection populations derived from the FEJ regime do not experience any selection for rapid development, with one regime experiencing the same duration of breeding time as the FEJ, and the other, a longer one. Since these two new regimes differed in the time available to flies for breeding, I was able to explore the effects of only this selection pressure in reducing inter-locus sexual conflict levels in the populations. The term relaxed selection is usually applied to artificial selection experiments, where the reproducing adults are no longer chosen by the experimenter in each generation (Falconer and Mackay 1996), as is being done in this case (see next section). I will be using *relaxed selection* in place of reverse selection subsequently since the former term describes the study populations more accurately.

Maintenance Protocols of the Study Populations

The studies reported in this thesis were performed on sixteen outbred *D. melanogaster* populations that have a common ancestry. Four of these populations were selected for faster pre-adult development and early reproduction and are referred to as the **FEJ**s (**F**aster developing,

Early reproducing, **J**B derived); the other four are ancestral controls, called the **JB**s (**J**oshi **B**aseline). The four control populations are descendants of a single wild-caught population of *D. melanogaster*, called the **IV** population (Rose and Charlesworth 1981). From this population, five **B** (**B**aseline) populations were derived, being maintained on a 14-day discrete generation cycle under constant light, 25 ±1^{o}C and high humidity on banana-molasses food (Rose 1984). After 360 generations from the derivation of B lines, another set of 5 populations were derived, one from each B population, called the **UU**s (**U**n-crowded as larvae, **U**n-crowded as adults) (Joshi and Mueller 1996) which were maintained on a 21-day discrete generation cycle, keeping all other aspects of maintenance the same. After about 170 generations of UU maintenance, the four JB populations were derived from the UU-1, 2, 3, and 5 and maintained in a manner similar to the UUs in banana-jaggery food medium, in our laboratory in Bengaluru, India (Sheeba et al 1998).

For each replicate JB population, about 60-80 eggs are collected into 8-dram glass vials (2.2 cm diameter × 9.6 cm height) containing 6 mL of banana-jaggery food. Forty such vials are collected per population. On the 12th day after egg collection, by which time all the flies in the JB vials have typically eclosed, flies are transferred to fresh food vials every alternate day. On the 18th day from egg collection, flies from all 40 vials are collected into a Plexiglas cage (25 cm × 20 cm × 15 cm) and provided with a Petri plate of food medium with a generous smear of live yeast and acetic acid paste. After three days of yeasting, the flies are allowed to lay eggs for about 18 hours on banana-jaggery food. Eggs from this food plate are then collected for the initiation of the next generation, thus cycling a generation every 21 days. Populations are maintained at a breeding adult number of 1500-1800 flies, under constant light, 25^{o}C ± 1^{o}C and about 90% relative humidity.

The four FEJ populations (first described in detail in Prasad et al 2000) are derived from the corresponding JB populations. The maintenance regime is similar, except that since the FEJs are selected for faster pre-adult development, only around the first 20-25% of the eclosing flies (per culture vial) are collected to be part of the breeding pool for the next generation. To obtain a sufficiently large population size (1500-1800 flies per population) 120 vials are set up per replicate population. Vials are checked for the requisite number of flies every two hours from the time of darkening of pupae, and once about 15 flies have eclosed in a vial they are collected into a Plexiglas cage. The population in the cage is then provided with a generous smear of live yeast and acetic acid paste on a banana-jaggery food plate for three days. Flies are then allowed to lay eggs for a duration of only one hour after the yeasting period on banana-jaggery food (to obtained similar aged eggs allowing efficient selection on development time), from which eggs are collected to initiate the next generation. The FEJ maintenance differs from that of the JBs in having only the earliest eclosing flies being part of the breeding pool (selection on faster development), and in having egg collection for the next generation about three days after eclosion (selection for early reproduction). Studies reported in this thesis were mostly conducted between generations 510-670 of FEJ selection, when the generation time of the FEJs had come down to 10 days due to selection on faster development and early reproduction, compared to their ancestral controls. Each of the four FEJ populations has been derived from one JB population (matched subscripts) such that, ancestrally, FEJ_i is more closely related to JB_i than to other FEJ populations. To account for ancestry, selected and control populations that share the same numerical subscript have been treated as random blocks in the statistical analyses.

From each FEJ population, two new populations were derived, around generation 600 of FEJ maintenance. Both were relaxed for selection for rapid development, collecting all the

eclosing flies to become part of the breeding pool. One set of populations was given about three days of breeding time after collection into a cage, and being supplied with a paste of live yeast over food medium to feed on. These populations were named the **R**elaxed for **R**apid development **F**EJ (**RRF**). Initially, as their development time was short, they finished eclosion by day seven from egg collection, were collected into a cage and yeasted for about three days before egg laying media plates were introduced for 18 hours, after which eggs were collected for the next generation. As the response to relaxed selection progressed, their development time increased and flies were collected into a cage on day eight.

In the other set of relaxed selection populations, eclosion was allowed to be completed, after which flies from each culture vial were transferred into a fresh food vial (vial transfer) as is done in JB populations. These were named the **R**elaxed **F**EJ (**RF**) populations. For the first seven generations, only a single vial transfer was done, on day nine from egg collection, and flies were collected into the cage on day 11, provided with additional yeast on food plates for about three days.Eggs for the next generation were then collected on day 14 after allowing egg lay for 18 hours. Progressively, as with the RRFs, the day of first transfer was postponed to day eight and then nine, and eventually two vial transfers were done every generation. Similar to the JB and FEJ, each population is referred to as RRF_i or RF_i, indicating the lineage of JB and FEJ that it has been derived from and is ancestrally more closely related to. A complete representation of the generation cycles for each regime (at the start of this work) is provided in Fig. 1.1.

Standardization and Collection of Flies for Assays

Since the selected and control populations experience different maintenance regimes, non-genetic parental effects can be confounded with traits that have evolved in different populations. It is, therefore, important to control for this by rearing both control and selected populations under similar conditions for at least one full generation before assaying any trait. In this case, standardization of the populations is achieved by collecting around 60-80 eggs per vial, with 40 vials per JB population and 60 vials per FEJ population (egg-adult viability of FEJs is lower than JBs, and 60 vials are needed to obtain the requisite adult population size of ~1500 individuals). Flies are collected into a Plexiglas cage as soon as eclosion is complete in all the vials, which corresponds to the 7^{th} day from egg collection for FEJs and the 11^{th} from egg collection for JBs. Since there is a development time gap between the FEJs, RRFs, RFs and JBs, egg collection is staggered for populations from different selection regimes to obtain freshly eclosed standardized adult flies on the same day. Eggs from the standardized flies are collected to obtain individuals for assaying any trait thereafter.

Work reported in this thesis is from studies on adult traits, and therefore age of the FEJ, JB, RF and RRF adults for assays was also synchronized by staggering egg collection from the standardized populations according to the difference in the pre-adult development time between populations from various selection regimes. Adult flies were housed in groups of 8-10 as early life exposure to perceived competition is known to affect male fitness components (Bretman et al 2009, Nandy and Prasad 2011).

Media Preparation

All study populations were cultured and reared on banana-jaggery food medium, the recipe for which is provided in Table 1.1.

About half to one third the amount of total water is heated along with agar and jaggery, allowing both to dissolve. Once the solution boils, a paste of yeast with the retained water and additional ethanol is added. Weighed and then peeled banana is also blended with some more of the retained water and added next, so as to not allow any lumps to form. Barley is added last, blended with the extra water listed in the recipe (Table 1.1). The mixture is allowed to come to a boil after which it is cooled down in a water bath to 65^{o}C before the preservative (sodium benzoate dissolved in ethanol) is added to it and thoroughly mixed. The media is then poured into vials or Petri dishes, as needed. The sequence of addition of ingredients is important to prevent lumps and allow proper integration of each into the medium.

Table 1.1 List of ingredients for banana-jaggery food medium.

Ingredients	**Quantity (1L of Media)**
Banana weighed with peel, which is then removed (g)	205
Barley (g)	25
Jaggery (g)	35
Yeast (g)	36
Agar (g)	12.4
Extra Water (mL)	180
Ethanol (mL)	22
Benzoate (g) + Ethanol (mL)	2.4 + 23

.

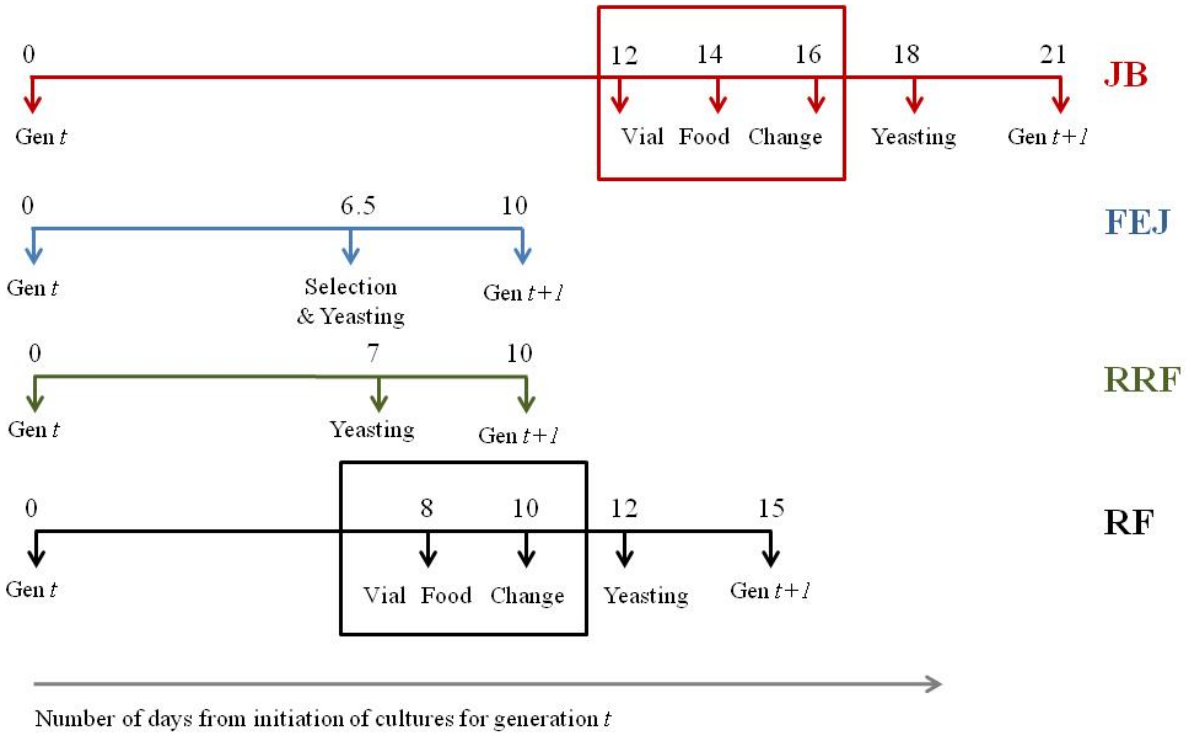


Fig 1.1 Representation of the discrete generation cycles of the various selection regimes showing the various stages of maintenance.

Chapter 2:

Differences between FEJ and JB Breeding Ecology

Introduction

Among sexually reproducing organisms, the interaction between the sexes for reproduction sets the stage for male-female co-evolution, and inter-locus sexual conflict is expected to be high in populations with large potential for sexual selection (Parker 1979, Arnqvist and Rowe 2013). Consequently, changing the degree of sexual selection is expected to affect inter-locus sexual conflict related traits in males and females. Numerous studies have looked at the consequences of experimental manipulation on male-male competition and female choice by subjecting the *Drosophila* model system to artificial selection in the laboratory, either by enforcing random monogamy (Holland and Rice 1999, Pitnick et al 2001, Crudgington et al 2005, Hollis et al 2014, 2017, Wensing et al 2017), or by manipulating the degree of polygamy by changing the operational sex ratio (Wigby and Chapman, 2004, Linklater et al 2007, Edwards et al 2010, Nandy et al 2013 a, b).

Monogamy adapted populations have been seen to evolve males that court less (Holland and Rice 1999), are worse off in competition for mating or fertilization with males from polygamous populations (Pitnick et al 2001, Wensing et al 2017), have reduced ability to suppress re-mating in females (Crudgington et al 2005), and have a smaller body size, testis size and disproportionately lesser sperm number (Pitnick et al 2001). Females from monogamy adapted populations, correspondingly, had reduced fitness when exposed to control or polygamy adapted males, in terms of both survival (Holland and Rice 1999), and reproduction (Crudgington et al 2005). There is also evidence of changes in expression patterns of genes relating to both intra- and inter-locus sexual conflict in monogamy versus polygamy adapted populations (Hollis et al 2014, 2017).

Similarly, females adapted to a male-biased adult sex ratio have better survivorship in the presence of males than do females from a female-biased line (Wigby and Chapman 2004), while males from the male biased populations show greater reduction of accessory glands in five successive matings, indicating greater investment of accessory gland proteins per mating by males adapted to a high competition environment (Linklater et al 2007). Nandy et al (2013 a, b) document the increased resistance to mate harm, both in terms of longevity and reproduction in females, and improved sperm competitive ability of males, belonging to their male sex ratio-biased selection regimes, as compared to the female sex ratio-biased ones. Moreover, since females bear a non-linearly increasing cost of re-mating (Kuijper et al 2006) the actual frequency of matings experienced in the life-time of females can drive both female resistance or avoidance behaviour (Rice et al 2006). These studies, thus, amply demonstrate the role of the sex ratio, breeding system and the competition experienced for mating and fertilization, in selecting for sexually antagonistic traits in *Drosophila sp*. The total adult life-span available for reproduction can, therefore, be an important determinant of the extent of sexual competition faced by individuals in *Drosophila* populations. Assuming similar probabilities of mating per unit time, longer time available for mating will result in higher competition among males, as life-time mating rate is expected to be higher in populations with longer breeding durations.

Life-time mating rate (Kuijper et al 2006), courtship rate (Partridge and Fowler 1990), and copulation duration (Gilchrist and Partridge 2000) can all be used as surrogates to establish differences in inter-locus sexual conflict. Kuijper et al (2006) have demonstrated, using a laboratory adapted *D. melanogaster* population, the direct fitness costs to females with increasing rates of re-mating, where the amount of sperm received remained the same under different mating rate conditions. In *D. melanogaster*, the duration of copulation also seems to be

twice that required for sperm transfer alone, with the remaining time probably spent in the transfer of accessory gland proteins (Gilchrist and Partridge 2000). Therefore, copulation duration itself can be indicative of the investment made by males towards their reproductive success. Finally, courtship in *D. melanogaster* is a pre-requisite for mating and presumably an important opportunity for females to assess the fitness of the male. Moreover, courtship is energetically intensive, consisting of a large repertoire of male behaviours, and bears a fitness cost to males (Cordts and Partridge 1996). An assessment of the courtship rate can, therefore, also provide information on the reproductive effort required of a male for successful mating.

Since the time from eclosion to final reproduction is quite different for the FEJ and JB flies (approximately three-four days vs. nine-ten days), and given that both are effectively semelparous due to the discrete generation maintenance system, matings that translate into realized fertilization success (i.e. fertilization of eggs that are laid on the day at which eggs are collected to initiate the next generation) can have a great impact on the effective competition experienced by males as well as mate choice exercised by females. That timing of mating can be a source of variation in reproductive success among *D. melanogaster* females has been demonstrated by Long et al (2010). Therefore, the pattern of mating and courtship with age is crucial for understanding the breeding ecology of the populations studied here. Assessment of overall mating and courtship rates alone, without information on fertilization success, would grossly ignore the details of male-female post-copulatory interactions and any trade-offs with other fitness requirements, like survival until the day of egg collection for initiating the next generation.

In this chapter, mating rate, courtship rate, copulation duration, time till sexual maturation (or mating latency of virgins) of females and pattern of fertilization success (for JB

populations) has been assessed. The intent has been to understand differences in these aspects of their reproductive ecology as a means of establishing whether the difference in age of reproduction in FEJs and JBs can act as a selective force in affecting levels of inter-locus sexual conflict.

Methods

For all experiments where mating and courtship rates were studied, standardization of populations was not done as the aim was to establish differences in their reproductive ecology as experienced in their regular maintenance regimes. Standardization was done for estimating time till reproductive maturity and mating duration of the FEJ, JB, RF and RRF populations as described in Chapter 1 (Pg 16). In the current chapter, only results from the FEJ and JB populations are presented, the RF and RRF results are reported in Chapter 5 of this thesis. Finally, to establish the most relevant window of mating in the JB populations, the JB and SE (scarlet eye mutant) populations were standardized in banana-jaggery food medium for a single generation before conducting the experiment. Our SE population was established by introgressing a spontaneously arisen scarlet eye-colour mutation in another *D. melanogaster* control population (MB3) which was derived from a population derived by mixing all the four JB populations, but maintained on cornmeal food medium. The SE population is a large, out-bred population containing ~87% of the MB3 genetic background.

Time till female maturation

To obtain adults of the same age at the beginning of the experiment, FEJ cultures were started about 60 hours after the JB cultures were initiated, from the standardized populations, accounting for the difference in their egg to adult development times (Satish 2010, A Mital and A Joshi, *unpubl. data*). The egg laying time given was reduced to 30 minutes so as to have a narrow eclosion distribution. At the beginning of eclosion, the first few flies emerging were discarded as a routine practice in order to collect flies from around the peak of the eclosion distribution, from both JB and FEJ cultures. I used flies that had emerged within the same one hour for the assay and set up 20 vials with a single pair per vial from each population with fresh banana-jaggery food medium. These vials were under continuous observation for a successful mating (recorded as a copulation event lasting at least three minutes) starting six hours after pairing of flies. Total copulation duration of the first mating was also noted. I used copulation as a read out of maturation of adult females, as our aim was to assess the time taken for flies to successfully start breeding from the time of eclosion. This usually occurs under presence of yeast supplement paste for FEJs and non-yeasted conditions for JBs. Therefore, both treatments were provided, with 20 vials per treatment, per population. A small smear of yeast-paste on the wall of the vial, close the food medium, constituted the yeasted environment. Total observation duration lasted for 56 hours, or until 80% of pairs per population, per treatment, had mated, whichever occurred first. Virgin males that were collected a day earlier from each population were used as the mating partners for the females under observation.

Courtship and mating rate

To assess the mating and courtship rates of flies as they were maintained in selection regimes I collected flies from JB and FEJ cultures, derived from the running cultures without undergoing any standardization, so as to assess breeding ecology differences that flies experience, including those due to non-genetic parental effects. The ecology includes the behaviour of the individuals in the population and therefore, for my objective, it was necessary to observe the flies in conditions very close to their regular maintenance protocol. In case of JBs, flies were collected from 12-day old cultures pairing five males and five females per vial (in fresh banana-jaggery food medium), setting up 10 vials per population. I chose five pairs to facilitate observations of courtship which were difficult to make with more than 10 flies in a vial. Since JB flies experience fresh food in vials every alternate day until day 18 from initiation of cultures, I set up observation vials similarly. On days 14 and 16 observation vials were again setup as described for day 12, except these were from adult holding vials that had previously been transferred to fresh food vials on days 12, and 12 and 14, respectively. Therefore, flies were kept in a fresh food vials with adult densities similar to those experienced in their regular maintenance until the day of observation. The same set of flies in vials were observed for two consecutive days (12-13, 14-15, 16-17) and then discarded. Since this entire vial phase of adult life is absent in case of the FEJ regime, I made these observations only with JB flies.

For observing flies in the last two days prior to egg lay, I set up 100 pairs of flies per Plexiglas cage (22 cm × 18 cm × 18 cm) with a plate of fresh food medium covered with a paste of live yeast, as in their regular maintenance. These cages were about half the volume of the regular population maintenance cages to approximate as closely as possible the adult density experienced by flies. The food plate, similarly, was also about half the surface area of the regular

food plates provided during routine population maintenance in cages. This is crucial since females tend to feed on yeast and crowd the surface of the food and yeast, which is also where most of the male-female interactions take place. I set up three replicate cages per population. Flies were first collected into a regular cage (from holding vials in JBs and culture vials in case of FEJs) and then 100 pairs were lightly anesthetized (using carbon dioxide) and transferred into each replicate observation cage. In case of FEJs, as in regular stock maintenance, only the first ~25% of the flies were selected to become part of the breeding pool.

I took three 'instantaneous' observations every four hours (corresponding to one time point) starting from day one of observation (i.e. day 12 from JB culture initiation, ~day 6.5 from FEJ culture initiation) noting the number of males observed to be performing a courtship behaviour (Hall 1968) towards a female and the number of pairs in copula. I assumed every pair observed in copula was an instance of a successful mating. Observations were conducted for 144 hours (six days) in the vials for JBs only and for 72 hours (three days) in Plexiglas cages for both JBs and FEJs. The three observations were averaged to obtain the courtship and mating rate for that time point. This was continued till the end of the experiment on day 21 from JB culture initiation and day 10 from FEJ culture initiation. FEJ cultures were staggered in time so that the cage observation for FEJ and JB were made during the same three-day period, thus ensuring similar environmental conditions. All observations were made in a quiet room with ample ambient light against a white background. The observation vials and cages were left undisturbed during the entire observation phase.

Estimation of Breeding Success of JB

Since JB flies experience a long adult life (about 9-11 days), I wanted to assess the reproductive success achieved by males that mate on different days after eclosion, since mating continues for the entire adult duration. For this, I used females from a similar population with a recessive eye colour mutation, scarlet eye (SE). The SE population is homozygous for this mutation and by allowing males from the wild type (WT) i.e. JB population to mate with SE females only during certain stages of their adult life, while housing them with SE males for the remainder of their lives, I was able to assess the reproductive success of the JB males, and which window of mating, if any, resulted in maximum offspring production.

I collected both SE and JB cultures for the experiment at the same time and on day 12 from their initiation, 24 vials were setup with 10 pairs of flies in each. I split the adult stage of the JB flies into four phases depending on the vial transfer and cage collection regime as is experienced by the JBs in their regular maintenance protocol. I introduced JB males with SE females on day 12 and housed them till day 14 after which the JB males were replaced with SE males using light carbon dioxide anesthesia, terming this Phase 1 (P1). Similarly, I housed SE females with JB males on days 14 and 15 (P2), 16 and 17 (P3) and 18, 19 and 20 (P4) after which the SE females were allowed to lay eggs (in individual vials) for 18 hours.

To account for anesthesia potentially affecting mating behaviour and therefore reproductive success of the males, I introduced fresh males into the breeding vials for all phases, irrespective of whether it called for a change in male type. Therefore for P1, fresh SE males were introduced into the breeding vials even on days 16 and 18, using light carbon dioxide anesthesia.

For P4, since JB flies experience a high yeast environment in the cage, I supplemented the food in the vial with a smear of live yeast paste.

I set up 30 egg laying vials for each phase, sampling five females from each of the breeding vials. The SE females were allowed to lay eggs (one female per vial) for 18 hours before being discarded and the cultures were maintained at 25^{o}C and constant light for the next 10-11 days until all flies had eclosed. I estimated the proportion of SE and WT progeny for each female and averaged this across females for a particular phase.

Statistical Analyses

I used mixed model analyses of variance (ANOVA) for all statistical analyses. Average time till maturation for a population, mean courtship and mating rate per population and proportion of WT offspring averaged across females per population were used as the response variables. Tukey's HSD test was used for comparing individual means and calculating all confidence intervals for post-hoc analyses. Since the data is of fractional nature, I also repeated each analysis with arcsine square root transformed values to check if there were any differences obtained in the significance of the fixed factors. Selection regime and yeasted/non-yeasted conditions for time till maturation and copulation duration, selection regime for courtship and mating rate assays, and phase for breeding success assay were treated as fixed factors in the analysis. Additionally, I checked within the JB population for significance in the differences between the vial and cage ecology in courtship and mating rates. All experiments were performed on four replicate populations of a selection regime. Replicate populations with the same numerical subscript share the same ancestry (FEJ_i and JB_i) and were therefore treated as

random blocks in the analyses. All analyses were carried out using STATISTICA™ using Windows Release 5.0B (Statsoft Inc. 1995).

Results

Mating and Courtship Rates

The differences between FEJ and JB lifetime mating (F=1020.928, P<0.001) and courtship (F=1372.439, P< 0.001) were statistically significantly (Fig 2.1, Table 2.1) with JBs showing considerably higher rates. When the mating and courtship rates only within the cage ecology (the last three days of mating of their effective life) were compared, courtship rates were still significantly greater for JB than for FEJ, but their mating rates did not differ significantly (Table 2.2). Although this was expected, the sheer magnitude of difference in the mating rates and courtship rates experienced by the FEJ and JB males over their effective lifetime revealed the potential for large differences in sexual selection operating in these populations. Moreover, although overall courtship rates were very low for FEJ, the results from the analysis of data from only within the cages indicated a very low courtship requirement for arousal of FEJ females for copulation, given that mating rates in the cage were very similar for JB and FEJ, despite courtship rates being much lower in FEJ (Fig 2.2). It is also worth noting that both courtship and mating rates drop by almost an order of magnitude for JB when only the cage ecology is considered.

Maturation Time and Copulation Duration

Maturation time of the JB and FEJ differed significantly (F= 149.885, P= 0.001), as did the copulation duration (F= 15.260, P= 0.029), with JB females maturing faster and mating for a longer duration than FEJ females (Figs 2.3, 2.4 Tables 2.3, 2.4). Surprisingly, although a lot of work has provided evidence for change in female mating behaviour with adult diet and nutrition, these results do not demonstrate any effect of yeasting on female maturation time or copulation duration. There was no significant selection-by-environment interaction either (Table 2.3, 2.4), indicating a lack of differential effect of yeasting on either FEJ or JB female mating behaviour. Female condition (being fed a protein rich diet as adults) is also known to affect male behaviour, in terms of increasing male courtship vigour through an increased attractiveness of the female, but any such effects on the FEJ or JB males, or differences in these effects, did not manifest in the traits assayed here, i.e., mating duration or mating latency of freshly eclosed virgin females.

Breeding Success

Results from this assay indicated an overwhelming last male precedence in breeding success in the JB populations, showing that last window of mating resulted in as many as 80% of eggs laid on day 21 from egg collection (Fig. 2.5). There was a significant effect of mating window (F=121.625, P= <0.001), with the final mating window of the last two to three days (cage ecology) yielding maximum fertilization success in terms of fertilization of eggs laid on day 21. The first two days of mating yielded the least success, significantly lower than that of the two middle windows of mating (corresponding to day 14 to day 18 from egg collection), with there being no significant difference between mating windows two and three, as revealed by pair wise

comparisons using Tukey's HSD (Fig 2.5, Table 2.5). The arcsine square root transformed data showed the same pattern of significance as the untransformed data.

Discussion

Effective Life-time Mating Rate

The results from the study of breeding ecology of FEJ and JB populations suggest that reducing the time available for mating reduces the overall competition experienced by the flies in their effective life-time. Although this by itself is intuitive, given the large difference in breeding time provided to FEJ and JB populations, I demonstrate here the extent to which this difference, coupled with selection for rapid development, can affect the sexual selection scenario in these populations. If we were to scale the mating rate results by an appropriate factor (maximum possible number of matings achievable), to obtain an expected number of life-time mating events, FEJ flies will be projected to experience around one mating event, living effectively monogamously (Fig.2.1 (A)). This is in comparison tothe six to eight times that a JB fly is likely to mate, using a similar scaling factor (Fig.2.1(A)), demonstrating a major, unintended consequence on potential degree of sexual selection of selection for time of reproduction in these populations. Given the large body of evidence for changes in male and female reproduction related traits with levels of sexual selection (see the Introduction to this chapter), I speculate that this difference in their selection regime itself could select for very low levels of manipulation and mate harm by the FEJ males. Consequently, the FEJ females would be selected to produce weaker, or no defense against post mating manipulation and harm, as a co-evolutionary response.

The effectively monogamous nature of the FEJ populations is strong support for its breeding ecology selecting for reduced inter-locus sexual conflict.

This reduction in requirement of high trait value of traits relating to inter-locus sexual conflict is also likely to affect the resource requirement and allocation in FEJ flies, either allowing them to be even smaller, thus further pushing the boundaries of rapid development, or allowing an increased investment in egg production (there is evidence for larger egg size (B M Prakash and A Joshi, *unpubl. Data*) and greater egg per unit dry weight of FEJ than JB (Prasad 2004, Ghosh-Modak 2009)), or both. Production of Acps is known to be costly to males (Chapman and Edwards 2011), and Nandy et al (2013, a, b) have demonstrated a possible trade-off between inter-locus sexual conflict related traits and other life-history related fitness traits such as fecundity and longevity (through body size) in *D. melanogaster* females.

Maturation Time and Copulation Duration

FEJ females were found to take far longer from eclosion to first mating, with their mating latency being about 10 hours more than that of JB (Fig 2.3). Compared to earlier work on the same populations at gen 70 of FEJ selection, this duration seems to have lengthened. One reason could be that many more generations of selection have elapsed since that observation (Prasad 2004), and the larger increase in maturation duration in FEJ (25 hours compared to 16 hours at generation 70) as compared to JB (15 hours compared to 12 in the earlier work) is consistent with this explanation. However, another possibility could be that I did not provide any competitor male as had been done earlier, which would induce greater mating effort on the part of males and reduce copulation duration (Bretman et al 2009, Nandy and Prasad 2011). Iconsider

this possibility as the mating latency was greater than observed by Prasad (2004) even for JB females, in this investigation.

This difference in protocol, however, cannot explain the overall shorter copulation duration seen in both JB and FEJ populations (Fig. 2.4). If anything, lack of a competitor male should prevent any interruption of copulation thus lengthening it. Moreover, mean maturation time of FEJ females seems to have increased disproportionately, by nine hours compared to that of JB, which has increased by three hours, and is expected to be an outcome of selection on the FEJ. At this point it is not clear why FEJ females would require even longer to mature. Admittedly, their development time has further reduced since gen 70, and possibly, some greater aspect of reproductive development has been postponed to adulthood, consistent with the argument made earlier (Prasad 2004) to explain why a similar extension of adult maturation wasn't observed in the ACO or ACB populations (Chippindale et al 1997a), which have the additional requirement as adults to reproduce as quickly as possible (about 24 hours) compared to the three days for FEJ, and had consequently not shown as significant a reduction in their development time as the FEJ.

The reduction in the copulation duration is also interesting in that shorter copulation duration would allow more mating in the limited time available, although this is an unlikely explanation given our mating rate results under the cage ecology (Fig. 2.1(A)). However, faster development and the subsequent body size reduction can result in further restrictions on resource allocation towards reproduction. FEJ females mate for around 15 minutes compared to JB females that mate for around 25 minutes, on an average (in yeasted or non-yeasted conditions) (Fig. 2.4). It has been suggested that in *Drosophila*, most of the sperm are transferred during the first half of mating (Gilchrist and Partridge 2000), the remaining time being spent in the transfer

of SFPs responsible for the female post-mating response. A smaller investment in these components of the *D. melanogaster* ejaculate would explain a shorter copulation duration in FEJ, also consistent with our argument that FEJ males are less manipulative and harmful to females, and that, therefore, females are less resistant (Mital 2013), supporting the idea of reduced inter-locus sexual conflict in the FEJ populations, compared to JBs.

Effective Life-time Courtship Rate

Courtship behaviours have long been theorized to be a consequence of either female preference (Fisher's runaway selection: Fisher 1915) or female resistance (chase away selection: Holland and Rice 1998). A male fruit fly, simply in order to mate, is required to manipulate/coerce a female into mating. This would require males to perform courtship behaviour which includes chasing, licking, wing extension and vibration (courtship song), and mounting (Spieth 1974). I found large differences in the courtship rates between the FEJ and JB flies (Fig. 2.1(B)), even if I only compare the cage ecology (Fig. 2.2 (B)), where the mating rates are similar (Fig. 2.2(A)). As in most animals, female fruit flies are courted extensively, and males need to arouse a female to a threshold value before the female becomes receptive to mating. It appears that FEJ females may have a very low arousal threshold, although I have not specifically tested for this.

Additionally, the short breeding duration might have selected the FEJ females to be less 'choosy' as at least one instance of copulation is necessary for them to have any offspring and, therefore, fitness, at all. A higher maturation time has further reduced the time available for mating for FEJ females (Fig.2.1 (A)), making lower courtship requirement and a shorter copulation duration even more selectively advantageous. Finally, this period between eclosion

and reproduction is also their only opportunity to feed and thereby accumulate additional resources for egg production while they are yeasted as adults, yeast being known to significantly boost fecundity in this lineage of *D. melanogaster* populations (Luckinbill et al 1985, Chippindale et al 1997b), and thus, make up for the lack of resources acquired during development. This can have imposed strong selection on FEJ flies to mate quickly, possibly by evolving a lower arousal threshold and, co-evolutionarily, lower courtship effort from males. Despite these compelling arguments, the most probable explanation is that the change in courtship is simply an outcome of developmental defects or changes in the process of development in the FEJ which have evolved a shortening of every developmental stage (Ghosh-Modak 2009).

My personal observations indicate that the type of courtship appears to have changed: FEJ males perform very little chasing behaviour, a part of courtship prominently seen in the JB populations. The reduction in chasing behaviour by FEJ males would also be consistent with it being a trade-off to rapid development. I have also observed the FEJ flies to be lethargic with regard to both flying and walking as compared to JB. Overall low rates of courtship by males and quick receptivity of females upon being courted can, therefore, be explained simply as austerity measures, or developmental anomalies. Ruhmann et al (2016) also report that *D. melanogaster* males keep maturing reproductively, especially in terms of increase in accessory gland size, for up to six days after eclosion, and younger males tend to have poor sperm competitive ability and perform less courtship, as compared to older, more mature males. This is consistent with FEJ male behaviour, since these males would be relatively young at the time of their first, and probably only mating, as compared to JB males.

A final line of argument for low courtship rates despite similar mating rates between FEJ and JB can be invoked assuming a chase-away selection model for female choice in *D. melanogaster* (Holland and Rice 1998, Gavrilets et al 2001). The chase away selection model suggests that female choice can evolve as a consequence of persistent male mating effort, which, in the event of being harmful to females (inter-locus sexual conflict), would give rise to female resistance towards such sensory manipulation but would be observed during male-female interactions as female bias/preference/choice for high courtship, i.e. some males that succeed in arousing the female with great courtship effort, despite the evolved resistance, would seem to be selected by her (Holland and Rice 1998, 1999). Any interaction between the sexes has to be detrimental to the fitness of one sex to trigger inter-locus sexual conflict, and persistent male courtship is known to reduce female survival in *D. melanogaster* (Partridge and Fowler 1990). If so, a reduction in inter-locus sexual conflict via low male-male competition (FEJ are effectively monogamous, Fig.2.1), would release the sexes from this co-evolutionary cycle, and appear as lack of female choice and male courtship effort. Therefore, it appears that low courtship rates for similar mating rates can be driven by female ecological constraints, development time reduction, or reduced male-male competition.

In the following section I discuss results from the breeding success experiment as evidence for selection for very high rates of courtship in the JB populations, especially in the cage ecology, when mating rates drastically drop.

Breeding Success

JB populations show significant last male fertilization success of eggs laid on day 21 from egg collection for the preceding generation, with mating that occurs in the last three days prior to egg lay resulting in a large (80%) paternity share (Fig. 2.5). If last male precedence for fertilization success (which has been well documented before (Fricke et al 2010, Manier et al 2010, and references therein)) occurs in JBs, then such a large paternity share can explain the very intense courtship and mating effort put in by JBs in the cage phase, i.e. the last window of mating, even though they secure many matings during the earlier vial stages of adult life. The overall low mating rate in the cage, however, is not surprising as females are expected to spend more time feeding rather than mating, having already secured the required sperm. Arguments for why JB, in general, court more than FEJ have been made in the previous section; here, I explore possible reasons for a disproportionate mating effort by JB males during the last stage of their adult life, in the absence of a correspondingly high mating success. This result also supports the existence of large variation in male mating success, a long-standing argument for stronger selection in males for exaggerated traits (Bateman 1948, Andersson 1994).

There is evidence that there can be strong selection on males for achieving mating close to egg lay, due to sperm competitive advantage or last male precedence (Fricke et al 2010,Manier et al 2010, and references therein). Service and Fales (1994) report that delayed reproductive senescence evolves in males in lines selected for late age of reproduction, as they get a fitness advantage through sperm competition. Strong selection for final mating effort in JB populations, which share a common lineage with the stocks used by Service and Fales (1994), is plausible, and consistent with expectation. As there is little mating that occurs in the cage, reproductive success is likely determined more by chance at this stage, strongly selecting for

males to attempt mating. This will impose increased selection on JB females to evolve greater resistance to harassment by males, a selection in the direction of increased inter-locus sexual conflict following the chase-away selection theory discussed above.

There is a growing body of work directed at understanding the details behind competitive fertilization success, thus explaining last male precedence, including non-sperm components of the ejaculate (Harshman and Prout 1994), along with sperm quality (Lüpold et al 2012) and cryptic female choice. In this work, I did not attempt to investigate methods by which last male precedence may exist in JB females as it was not crucial for answering the question posed in Chapter 1. In fact, the greater paternity share from matings that occurred in the final mating window might be explained by the fact that females would have already laid eggs that were fertilized by earlier matings, before the eggs to start the next generation were collected. However, this experiment does demonstrate that increased selection is experienced by JB populations for inter-locus sexual conflict related traits, as males experience very intense competition, especially in the final stage of their operative adult life, unlike FEJ males, with or without the operation of last male precedence.

Conclusion

Having a shorter pre-adult development time, and therefore a smaller adult body size, can give rise to major changes in adult mating behaviour. Since smaller *Drosophila* males have been shown to be less harmful to females post mating (Pitnick and Garcia-Gonzalez 2002), this itself could trigger a reduction in overall inter-locus sexual conflict levels in FEJ, as also argued earlier

by Mital (2013). Nevertheless, the adult flies of the FEJ populations also experience conditions conducive to reduction of inter-locus sexual conflict, at least in part due to the differences in their breeding ecology. This begs the question as to what role, if any, has the difference in time of reproduction, by itself, played in the reduction of inter-locus sexual conflict in the FEJ populations? Would these differences in the adult stage of their life-history give rise to differences in inter-locus sexual conflict if selection for rapid development were removed?

The primary objective behind conducting this set of experiments was to establish whether or not selection for time of reproduction acts as a selection force affecting levels of inter-locus sexual conflict in the FEJ and JB populations. These results suggest that this aspect of selection might be playing such a role, and forms the basis for further work reported in this thesis. Investigations into the extent to which the two selection pressures (selection for reduced development time and sexual selection, respectively) contribute to the changes in male and female reproduction related traits in the FEJ populations, as compared to JB, are discussed in the subsequent chapters.

Tables

Table 2.1 Summary of results of a one way ANOVA each for lifetime mating and courtship rates. Main effects of selection regime for the two traits are shown. In this design, random factors and interaction effects cannot be tested for significance and have been left out for brevity.

Effect	**df**	**MS**	***F***	***P***
Mating Rate	1	0.5502	1020.928	< 0.0001
Courtship Rate	1	120.2025	1372.439	< 0.0001

Table 2.2 Summary of results of a one way ANOVA each for mating and courtship rates, only in the cage ecology. Main effects of selection regime for the two traits are shown. In this design, random factors and interaction effects cannot be tested for significance and have been left out for brevity.

Effect	**df**	**MS**	***F***	***P***
Mating Rate in Cage	1	0.0001	4.163	0.1339
Courtship Rate in Cage	1	0.0010	910.352	<0.001

Table 2.3 Summary of ANOVA results for maturation time with selection regime and environment (yeasted or non-yeasted) as fixed factors. In this design, random factors and interactions with the same cannot be testes and have been left out for brevity.

Effect	**df**	**MS**	***F***	***P***
Selection	1	535.6813	149.885	0.0011
Environment	1	1.6626	0.203	0.6823
Selection × Environment	1	1.0137	0.003	0.9542

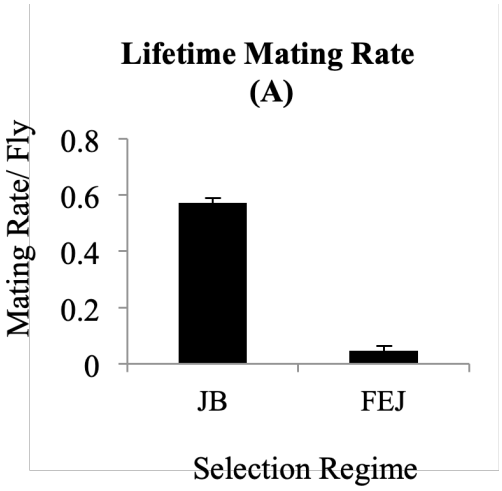
Table 2.4 Summary of ANOVA results for copulation duration with selection regime and environment (yeasted or non-yeasted) as fixed factors. In this design, random factors and interactions with the same cannot be tested for significance and have been left out for brevity.

Effect	**df**	**MS**	***F***	***P***
Selection	1	143.8742	15.260	0.0297
Environment	1	0.6389	0.646	0.4802
Selection × Environment	1	0.0867	0.0326	0.8680

Table 2.5 Summary of one way ANOVA results for breeding success in JB (proportion of wild-type) with mating window as a fixed factor. Since random factors and interactions with them cannot be tested for significance in this design, they have been omitted for brevity.

Effect	**df**	**MS**	***F***	***P***
Mating Window	3	0.5327	121.625	<0.001

Figures



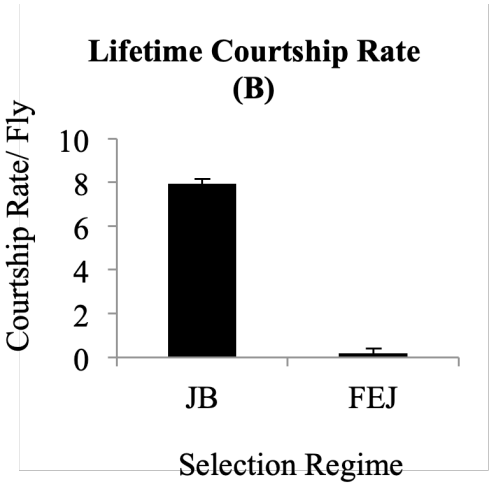
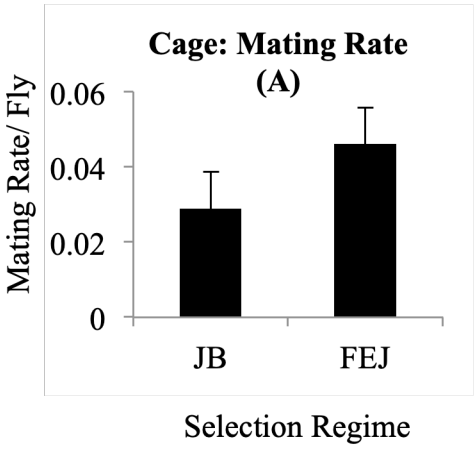


Fig 2.1 A) Mean lifetime mating rating averaged across the four replicate populations of JB and FEJ. **B)** Mean lifetime courtship rate averaged across four replicate populations of JB and FEJ. Error bars are 95% confidence intervals around the means.



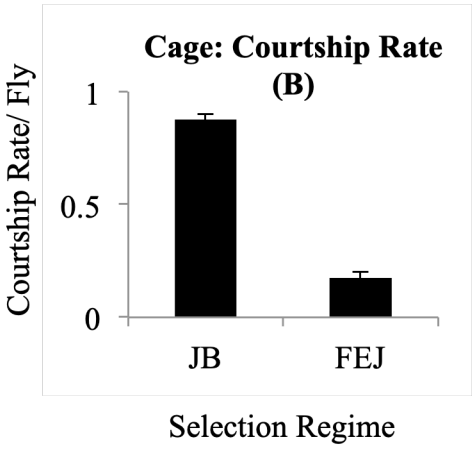


Fig 2.2A) Mean mating rate in the cage, or the final three days of adult life, averaged across four replicate populations of JB and FEJ. **B)** Mean courtship rate in the cage, averaged across the four replicate populations of JB and FEJ. Error bars are 95% confidence intervals around the means.

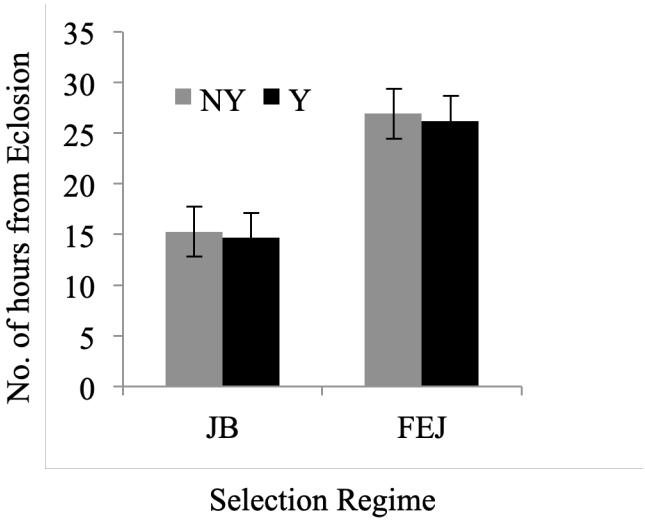


Fig 2.3 Mean maturation time, or mating latency of virgins from eclosion, averaged across four replicate populations of JB and FEJ, under yeasted (Y) and non-yeasted (NY) conditions. Error bars are 95% confidence intervals around the means.

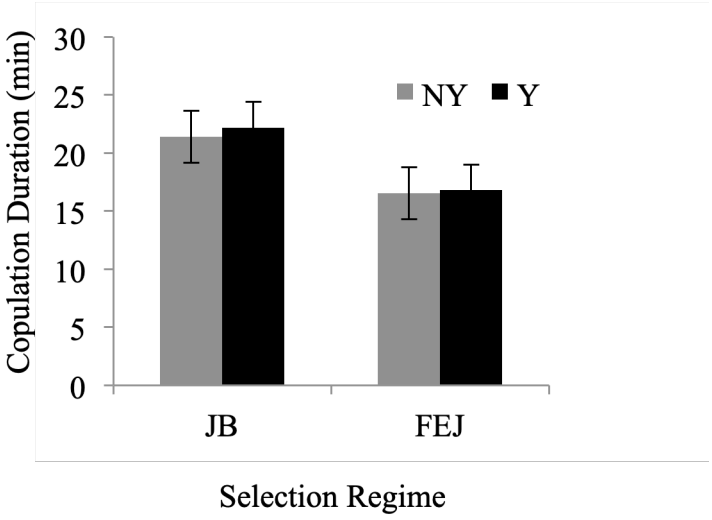


Fig 2.4 Mean copulation (mating) duration of the first mating, averaged across four replicate populations of JB and FEJ, under yeasted (Y) and non-yeasted (NY) conditions. Error bars are 95% confidence intervals around the means.

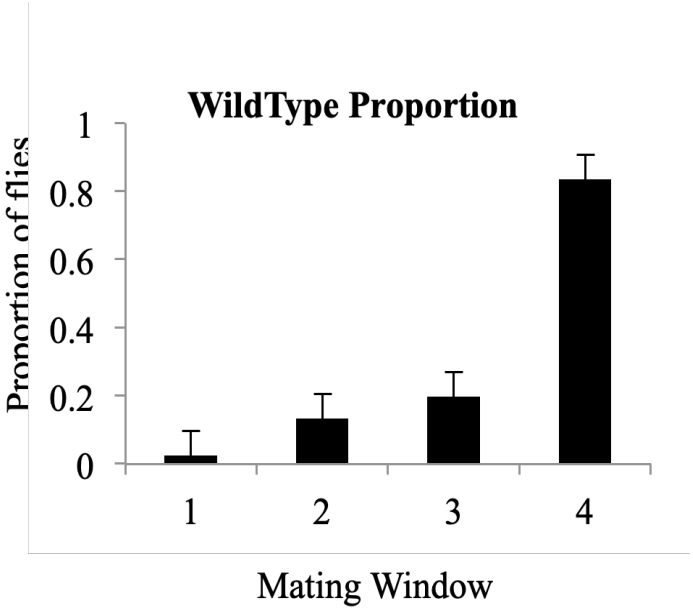


Fig 2.5 Mean proportion of JB flies in the breeding success assay that resulted from eggs laid on day 21 from egg collection, averaged across four replicate populations, showing success in various mating windows. Error bars are 95% confidence intervals around the means.

Chapter 3:

Direct Response to Relaxed Selection in RF and RRF

Introduction

The effect of the degree of polygamy, or level of sexual selection, on traits related to inter-locus sexual conflict has been demonstrated in a number of studies (see references Chapter 1, Chapter 2: Introduction). This prompted an exploration of the effects of selection for early reproduction in the FEJ populations, compared to their controls, as a possible explanation for the reduction in inter-locus sexual conflict levels, leading to incipient reproductive isolation, (Ghosh and Joshi 2012, Mital 2013). Based on the results described in Chapter 2, and the arguments presented therein, there is evidence to suggest that selection on time of reproduction could be facilitating the evolution of lower levels of inter-locus sexual conflict in the FEJ populations.

I chose to use an experimental evolution approach to investigate the potential role of early reproduction in bringing about an evolutionary reduction in the level inter-locus sexual conflict in the FEJ populations. From the FEJ populations, two additional selection regimes were derived, each selected for either early reproduction (about three days, as in the FEJ) or later reproduction (around eight days), but both relaxed for selection on rapid development. The release from the constraint of developing fast would allow these populations to diverge only with respect to the time available as adults for breeding. These populations are RRF_i (relaxed for rapid development, selected for early reproduction) and RF_i (relaxed for both rapid development and early reproduction). The details of the maintenance and selection protocol of these populations have been presented in Chapter 1 (Fig 1.1).

This method of exploration can also shed light on additional causes of population divergence, such as random genetic drift. The JB lineage of *D. melanogaster* has been maintained in the laboratory for at least a thousand generations, around 350 generations since

they were brought to this laboratory and re-named the JB (Fig 1.1). The FEJ populations had undergone selection for around 550 generations (at the time of initiation of the new relaxed selection regimes). Given the strong selection imposed on FEJs for a large number of generations, alleles responsible for rapid development, and others that might confer a fitness advantage to flies under these conditions of selection, can approach fixation. Theory also suggests that number of generations equal to twice the effective population size is long enough to expect some evidence of such fixation events due to drift itself, under consistent conditions of maintenance (Falconer and Mackay 1996). On populations maintained under a similar absolute population size, belonging to the same lineage, and maintained on a similar protocol (albeit without intended selection on development time), the probable effective population size has been estimated to be <1000 (Mueller et al 2013). Therefore, given the ~ 1500 generations of laboratory maintenance of FEJs, together with the imposition of strong, directional selection for ~550 of those generations, suggests that fixation of alleles at various loci due to selection or random genetic drift in these populations could have occurred. It must be noted however, that the effectively monogamous nature of the FEJ populations (Chapter 2) would partly ameliorate such effects by tending to equalize family sizes, thereby increasing the effective population size. Nevertheless, the rate of response of traits to a relaxation of selection pressures would allow us to explore this aspect of the populations further, chance being a crucial cause of trait divergence, and worthy of consideration as it bears heavily on interpretations of results from such selection experiments (Archer et al 2003, Joshi et al 2003, Phelan et al 2003). Further, since populations undergoing relaxed selection for rapid development and early reproduction have been derived from FEJs once before (Satish 2010), starting at FEJ generation 286 (compared to FEJ

generation 550 for RRFs and 557 for RFs in this instance), a comparison of rate of response to the relaxed selection in these two sets of populations could be illuminating.

Since the RF and RRF regimes experience no selection for faster development, I expect development time, and consequently body size at eclosion, to increase in these populations compared to the FEJs, given the significant fitness costs of rapid development in the FEJs reported earlier (Prasad et al 2000, 2001, Prasad 2004, Shakarad et al 2005, Modak et al 2009). Furthermore, since the effective adult lifespan is different for the RF and RRF populations, I also expect this response to occur at different rates, with increase in pre-adult development time and body size to happen faster in the RFs than the RRFs. The longer adult duration in the RFs might require more resources for the flies to survive longer, and pre-adult development time and body size at eclosion are known to be strongly correlated with longevity in this lineage of flies across laboratories (Prasad 2004, Chippindale et al 2004).

In this chapter, I report the direct response to relaxation of selection for rapid development in the RF and RRF populations, checking pre-adult development time, stage specific development time, and body size at eclosion. This has been done in order to establish their response to the change in selection pressure before proceeding further with the investigation of inter-locus sexual conflict. Consistent with past work on other, similar populations (Zwaan et al 1995a, Nunney 1996, Chippindale et al 1997a) and the same populations (Prasad et al 2001), pre-adult development time was strongly correlated with adult size at eclosion. A comparison with the previous instance of keeping RFs (Satish 2010) shows a much slower response to relaxation in the present study, indicating a possible decrease in additive genetic variation in sFEJs for development time over the 300 or so intervening generations of FEJ selection between the two relaxed selection studies.

Methods

Pre-Adult Development Time

After generations 304, 591, 22 and 42 of JB, FEJ, RF and RRFs, respectively, a pre-adult development time assay was conducted as the first check for the response to relaxed selection on RF and RRFs. All four populations were standardized, one block at a time, for logistic reasons. These standardized populations were provided with live-yeast paste for about three days. A 'dummy' egg collection plate was placed in each cage for an hour to allow more mature eggs retained by females to be laid. These were then replaced with another egg collection plate (sterile, in terms of eggs) of double agar food medium (same recipe as the banana-jaggery food medium with twice the quantity of agar, sugar instead of jaggery, and yeast, only), with a larger vertical surface area for egg lay. The vertical surfaces were created by cutting the egg lay media along the side of the Petri dish, exposing the vertical surface once the extra media was removed. After an hour, eggs from each population cage were scraped onto a sterile agar plate (only agar and water with preservative, following the banana-jaggery food recipe) and approximately 70 eggs were collected, using a fine paint brush and water, into food vials with 6 mL of banana-jaggery food medium. Ten such vials were prepared for each population. The mid-point of the one hour main egg lay window was noted as the set-up time. Around six days after the set-up, vials were checked every two hours for eclosion of flies and this continued till all eclosion was complete (no darkened pupae were observed in the vial or no new flies had eclosed in six hours). At each check, number of eclosing flies, and their sex, was recorded for each vial.

Stage-Specific Development Time Experiment

This experiment was conducted after generations 312, 608, 35 and 60 of JB, FEJ, RF and RRF, respectively. For the stage-specific development time experiment, standardization and 'yeasting' of standardized populations was done as described above. Instead of 10 vials per population, 60 vials were set up for each with ~70 eggs per vial, as described above. Based on a stage-specific development time experiment conducted earlier on the FEJ and JB populations (Ghosh-Modak 2009), four vials were sacrificed starting at 52 hours from the set-up time for FEJ, RF and RRFs, and 56 hours from set up for JBs, every two hours till 76 hours and 80 hours from set up, respectively. At each check point, vials were sacrificed by pouring extremely hot water into the culture vials, thus killing and halting further development of the larvae. These vials were then preserved at -20^{o}C for further investigation.

Once pupation began, vials were no longer sacrificed but the number of pupae in each vial was noted, and each one marked on the wall of the vial with a pen. Checks were conducted every four hours for pupation and eclosion with the remaining eight vials. In some cases, due to unavailability of eggs from the populations during set up, only 56 vials were set up such that only four vials were used for pupal and adult checks. Vials preserved from each of the larval checks were thawed and 30 larvae from each culture were sampled, using a fine, soft paint brush. These were arranged on a glass slide and mounted with a cover slip using water. The cover slip was gently pressed in order to squash the larvae and enable two-dimensional viewing of the mouth parts.

Using the number of teeth on the mouth hooks as the marker, different larval stages were identified from each check point and for each population, and the number of larvae in either

second or third instar, or in the second-third instar transition stage was noted. The median time for the onset of the third instar and pupal stage was calculated.

Dry weight at Eclosion

From each of the two experiments described above, the eclosing flies were separated by sex and stored in vials at -20^{o}C for dry weight estimation immediately after each check. From each population, 25 males and 25 females were sampled; five each were placed either in a glass vial covered with aluminum foil, with perforations on the cap to allow drying. These were then placed in a 60/70^{o}C hot air oven for 36 hours for drying. Flies from each vial were then weighed three times on a Sartorius (CP 225D) fine balance, and an average of the three readings was taken as the weight of each batch of five flies.

Statistical Analyses

For each population and each sex, the average egg-to-adult development time in hours, and dry weight per fly were calculated and three-way, mixed model analyses of variance (ANOVA) were performed with block as a random factor, and sex and selection regime as fixed factors. Similarly, egg-to-third instar duration, third instar duration and pupal duration were calculated for each population and a two-way, mixed model ANOVA was performed with selection regime as a fixed factor and block as a random factor. Tukey's HSD test was used for comparing individual means and calculating all confidence intervals for post-hoc analysis. All analyses were run on STATISTICA^{TM} using Windows Release 5.0B (Statsoft Inc. 1995).

Results

Pre-Adult Development Time

The two sets of experiments, one conducted around generation 40 of RRF and 20 of RF selection, and the other at generation 60 of RRF and 35 of RF selection revealed a response to the relaxation of selection pressure for rapid development, with both these sets of populations being significantly slower at developing compared to FEJ (Tables 3.1, 3.2, Figs 3.1, 3.2). This response, however was slower than that seen in the RF studied earlier by Satish (2010) which were relaxed for ~50 generations: in that study, within 25 generations, RFs were taking about 210 hours to complete pre-adult development, while the results from the present assays show RFs still taking 160-170 hours to complete development even after 35 generations of relaxation (Fig 3.2). In fact, the second assay of pre-adult development time showed RFs and RRFs taking less time, despite continued relaxation for rapid development, compared to the first round of assaying. This is probably due to environmental differences as the overall development time across all selection regimes was reduced, as much as about 10 hours for FEJ, RF and RRF and about five hours for JBs. These results show that a very small percentage of the ancestral trait value was recovered despite more than 35 generations of relaxed selection. Satish (2010) also found the response to have plateaued after 25 generations of relaxation, with no further increase even after 50 generations of RF maintenance. It appears that this stage of plateau was reached by the new RFs around 20 generations or before, and their development time increased only by about 10 hours in that time.

Although RRFs had undergone many more generations of relaxed selection, having a shorter generation time, their development time was significantly less than that of the RFs in

both assays (Figs 3.1, 3.2). The lack of a significant main effect of sex in the first assay was unusual (Fig 3.1), although I did find the effect of sex to be significant in the second assay (Table 3.2). Overall, females developed faster than males, as is expected in *D. melanogaster*.

Stage-Specific Development Time

Results from this assay demonstrate that the egg-to-third instar and third instar durations were significantly greater for RRFs and RFs compared to the FEJ (Fig 3.3 A, B), but this was not the case for pupal duration (Fig.3.3 C). These differences in egg-to-third instar and third instar durations accounted for most of the total development time difference between the FEJ and the two relaxed selection regimes. Earlier work on the FEJs revealed that the second instar duration is the last to respond to selection for rapid development, while the third instar is the first (Prasad 2004, Ghosh-Modak 2009). It was speculated that the second instar probably is an important stage in the development of the fruit fly, while third instar, which largely consists of the resource accumulating stage after attainment of critical mass for pupation, is more plastic and reduction in this stage will therefore contribute substantially to reduction in overall development time without significantly compromising crucial developmental processes (Prasad 2004). In this case, the recovery of longer development time through the larval stages, with no significant difference in the pupal duration is very telling. It confirms the likely importance of the larval stages for development and might also indicate a lack of genetic variation in the FEJ population at this stage of selection for pupal duration, but not for the larval stages.

These findings are in contrast to the results obtained by Satish (2010) wherein both larval and pupal stages showed a similar contribution to overall increase in pre-adult development time in the relaxed selection populations, with larval durations contributing slightly more (~56%).

Dry Weight at Eclosion

The results of dry weight measured at eclosion were consistent with expectation, with the RRFs and RFs weighing slightly more than the FEJ and significantly less than JB flies (Figs 3.4, 3.5, Tables 3.4, 3.5). Females weighed more than males, although in the first assay this effect was not significant (Table 3.4), as was the case for the effect of sex on pre-adult development time. The overall dry weight also was lower for all populations in the second assay, as was pre-adult development time. These differences in significance levels as well as overall trait values are likely to be due to environmental differences in temperature or humidity from assay to assay, as development time and weight at eclosion are known to be highly sensitive to such environmental factors. Overall, the response to relaxation of selection for rapid development was reflected in the dry weights of the flies at eclosion, but no significant difference was seen between the RFs and RRFs, although RFs showed slightly longer development times than RRFs.

Discussion

The RF and RRF pre-adult development times were found to be higher than those of FEJs, this difference increasing with continued relaxation of selection on development time between the two assays (Figs. 3.1, 3.2) After 40 generations of RRF and 20 generations of RF selection, the

pre-adult development time was about eight and ten hours more than that of FEJ, respectively, while after 60 generations of RRF and 35 generations of RF selection, these differences went up to around 15 hours and 20 hours, respectively. In the previous instance of RF selection (Satish 2010), after 25 generations of relaxation, RFs took 210 hours to complete development. Since FEJs were not assayed along with the RFs at the time (Satish 2010), the exact difference is not known. However, FEJs were taking around 160 hours to complete development from egg stage before the RFs were started by Satish (286 generations of FEJ selection), and there has been no reduction in their development time any further since. Therefore, by the time 25 generations of the earlier RF selection had elapsed, the RF populations were taking about 50 hours longer to develop compared to the FEJs, although this reached a plateau and no further increase in RF development time was seen after this. Since there is a cost to developing fast, in terms of pre-adult survivorship and body size at eclosion, (Chippindale et al 1994, Zwaan et al 1995a, Nunney 1996, Prasad et al 2001), the slower response to relaxed selection seen in the present study could be due to a further loss in additive genetic variation for development time in FEJs in the intervening generations. This is not surprising since FEJs have undergone strong directional selection for almost 300 more generations since the previous instance of RF initiation. This finding is consistent with the erosion of genetic variation for traits under strong directional selection (Falconer and Mackay 1996).

This is in contrast with the comparison made by Satish (2010) between the FEJ and the ACO populations (Teotonio and Rose 2000, Teotonio et al 2002) comparing two reverse selected regimes that were both derived from faster developing selection regimes, which was done in order to trace the rate of response back towards the ancestral trait values. Despite FEJs having undergone an additional 100 generations of selection compared to the ACOs, at the time of

starting the reverse selection populations, the trajectory of RF response was parallel to that of the RACOs, with ~75% of ancestral trait value being recovered in ~50 generations. The rate or degree of response to relaxed selection can be affected by reduced genetic variation, new patterns of epistasis or genotype-by-environment interactions (Teotonio and Rose 2001). Since I have not compared two different sets of selected populations, but the same set separated by ~300 generations of forward selection, the more likely causes of seeing much slower reverse evolution trajectories are new patterns of epistasis or the loss of additive genetic variation for development time during the additional 300 generations of forward selection. With most of this increase occurring during the larval stages (Fig 3.3 A, B), and very little contribution of the pupal stage (Fig. 3.3 C), unlike the results found by Satish (2010), it appears that genetic variation for pupal duration might have been lost in the FEJs by the time of the present study.

Results from the first development time assay also showed no significant effect of the sex-by-selection interaction on development time or dry weight at eclosion, but dry weight at eclosion in the second assay did reveal greater differences between males and females in JBs compared to FEJs, consistent with earlier work (Prasad et al 2000).The most likely cause of this is an effect of the assay environment, as development time is known to be affected by minor environmental changes. A significant reduction in sexual dimorphism for egg-to-adult development time and body size at eclosion was a prominent feature of forward selection for rapid development (Prasad et al 2000), reflecting that either the means of resolution of intra-locus sexual conflict were unavailable, or differences in optimal trait values for the two sexes were reduced (Rhen 2000). Relatively recent work has demonstrated the feminization of gene expression patterns in *D. melanogaster* populations maintained under random monogamy compared to populations of the same origin but maintained under polygamy (Hollis et al 2014).

A reduction in levels of sexual selection is expected to give rise to similar 'evolutionary interests' for the two sexes, possibly reducing ontogenetic conflicts (Prasad et al 2006). Therefore, the effectively monogamous adulthood of FEJs might have contributed to the convergence of the optimal body sizes of the sexes seen in FEJs (Fig 3.5). These reduced differences between sex-specific body sizes might also be due to selection for extremely rapid development where both males and females are at their natural selection limits for rapid development, and any scope for expressing sex specific optima is eliminated.

It is worthwhile to note that the protocol used for these development time assays was slightly different from those used earlier in this lab. I chose to use an egg density of ~70 eggs/ 6 mL of food compared to 30 eggs/6 mL of food. This increase in egg density does not give rise to any larval crowding related effects; it is low enough to prevent any limitation of food and, therefore, any scramble competition. This change from established protocols was made in order to estimate development times in assay conditions resembling culture conditions as closely as possible. Moreover, this was useful during the stage specific development time assay where I was able to successfully sample 30 larvae per culture for every time point, without any compromise on sample size which might have occurred due to accidental damage of larvae during removal from the food medium.

Both RFs and RRFs showed a clear increase in pre-adult development time compared to the FEJs, with RFs being even slower developing than the RRFs, despite fewer generations of relaxation (Fig 3.1, 3.2). Selection for a longer adult life probably selects RFs indirectly for greater resource acquisition and body size i.e. *reverse* selection (longer development time) and not just relaxed selection, as in the RRFs, resulting in the two new regimes experiencing different degrees of selection.

To conclude, the response to relaxation of selection on development time was established in the assays reported in this chapter, and the subsequent chapters investigate differences between RFs and RRFs in terms of sexually selected and inter-locus sexual conflict related traits, exploring the possible role of differences in timing of reproduction in mediating the evolution of these traits.

Tables

Table 3.1 Summary of mixed model ANOVA for pre-adult development time with selection regime and sex as fixed factors and block as a random factor, after generations 20 of RRF and 15 of RF selection. This design does not permit testing of significance for random factors and interactions and these results have been left out for brevity.

Effect	**df**	**MS**	***F***	***P***
Selection	3	8454.195	163.216	<0.001
Sex	1	17.589	45.010	0.006
Selection x Sex	3	0.4378	0.411	0.748

Table 3.2 Summary of mixed model ANOVA for pre-adult development time with selection regime and sex as fixed factors and block as a random factor, after generations 60 of RRF and 35 of RF selection. This design does not permit testing of significance for random factors and interactions and these results have been left out for brevity.

Effect	**df**	**MS**	***F***	***P***
Selection	3	10775.0166	70.360	<0.001
Sex	1	43.2682	82.967	0.002
Selection x Sex	3	0.0597	0.223	0.877

Table 3.3 Summary of one way ANOVAs with selection regime as a fixed factor performed on replicate population means for various developmental stages, after generations 60of RRF and 35 of RF selection. This design does not permit testing of significance for random factors and interactions and these results have been left out for brevity.

Effect	**df**	**MS**	***F***	***P***
Selection				
a) Egg-to-third instar	3	138.9100	60.873	<0.001
b) Third Instar	3	620.1373	75.545	<0.001
c) Pupal	3	1494.01	24.088	<0.001

Table 3.4 Summary of mixed model ANOVA for dry weights at eclosion with selection regime and sex as fixed factors and block as a random factor, after generations 40 of RRF and 20 of RF selection. This design does not permit testing of significance for random factors and interactions and these results have been left out for brevity.

Effect	**df**	**MS**	***F***	***P***
Selection	3	80.3914×10^{-9}	34.161	<0.001
Sex	1	30.838×10^{-9}	9.714	0.052
Selection × Sex	3	2.2845×10^{-9}	1.090	0.400

Table 3.5 Summary of mixed model ANOVA for dry weights at eclosion with selection regimes and sex as fixed factors and block as a random factor, after generations 60 of RRF and 35 of RF selection. This design does not permit testing of significance for random factors and interactions and these results have been left out for brevity.

Effect	**df**	**MS**	***F***	***P***
Selection	3	$1.1667 \times 10^{\wedge-6}$	104.118	<0.001
Sex	1	$2.178 \times 10^{\wedge-7}$	2010.461	<0.001
Selection × Sex	3	$2.5008 \times 10^{\wedge-8}$	11.169	0.002

Figures

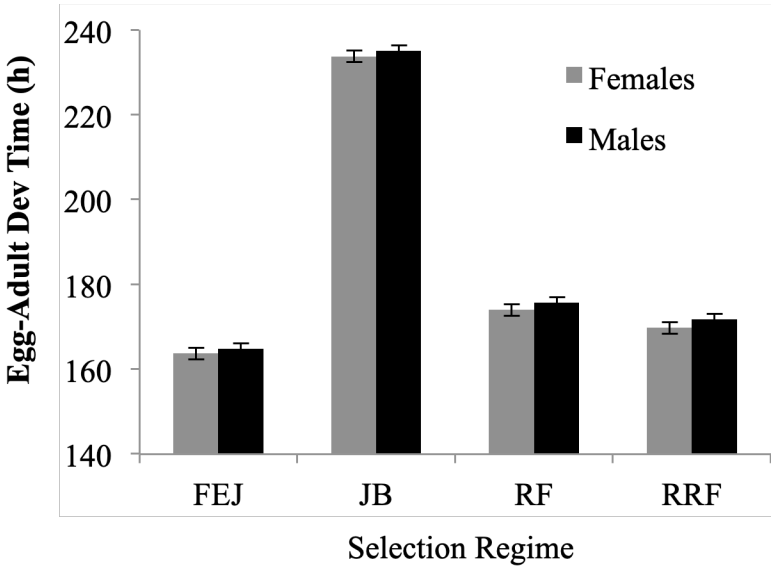


Fig 3.1 Mean pre-adult development time of FEJ, JB, RF, and RRF males and females, averaged across four replicate populations, after 40 generations of RRF and 20 generations of RF selection. Error bars are 95% confidence intervals around the means.

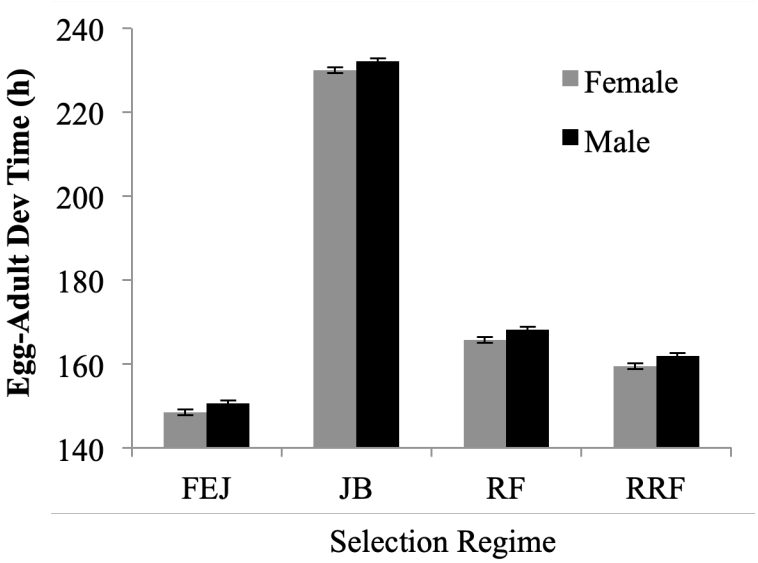


Fig 3.2 Mean pre-adult development time of females and males averaged across four replicate populations, after 60 generations of RRF and 35 generations of RF selection. Error bars are 95% confidence intervals around the means.

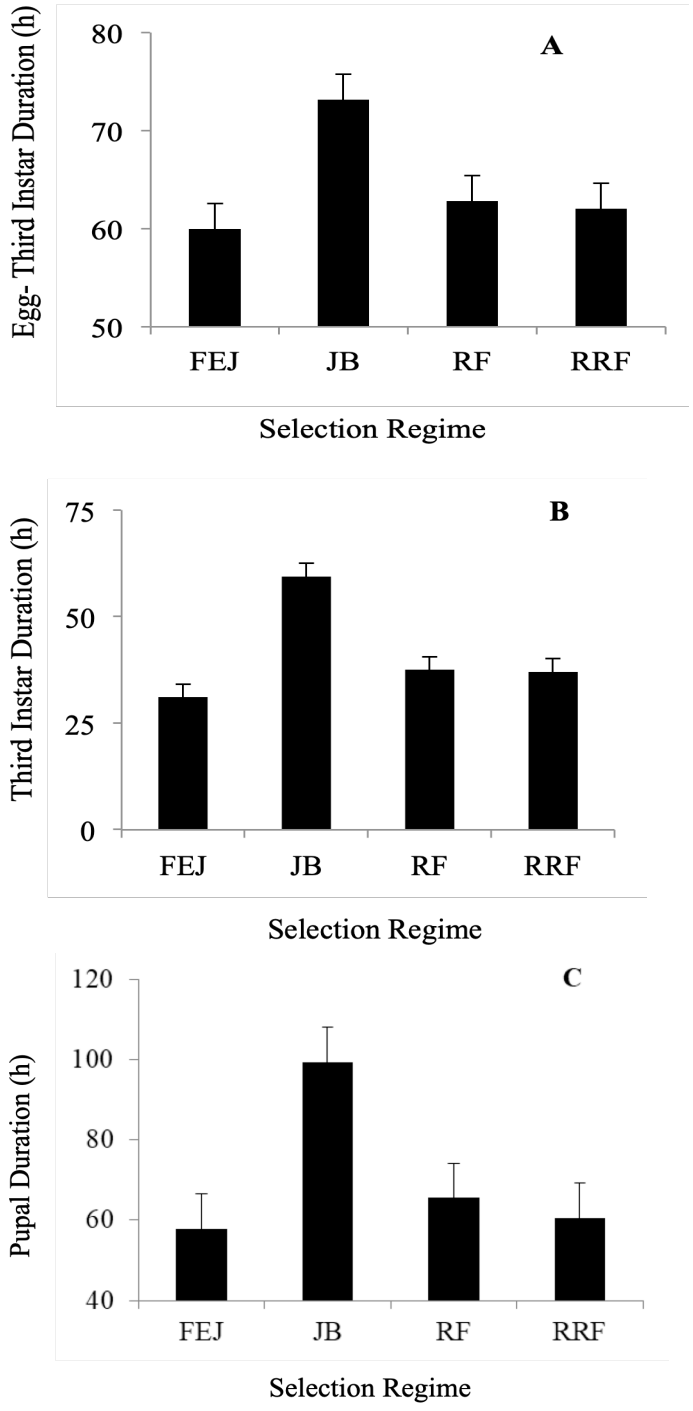


Fig 3.3 (A) Mean egg-to-third instar duration, **(B)** mean third instar duration, **(C)** mean pupal duration of FEJ, JB, RF, and RRF, averaged across four replicate populations, after 60 generations of RRF and 35 generations of RF selection. Error bars are 95% confidence intervals around the means.

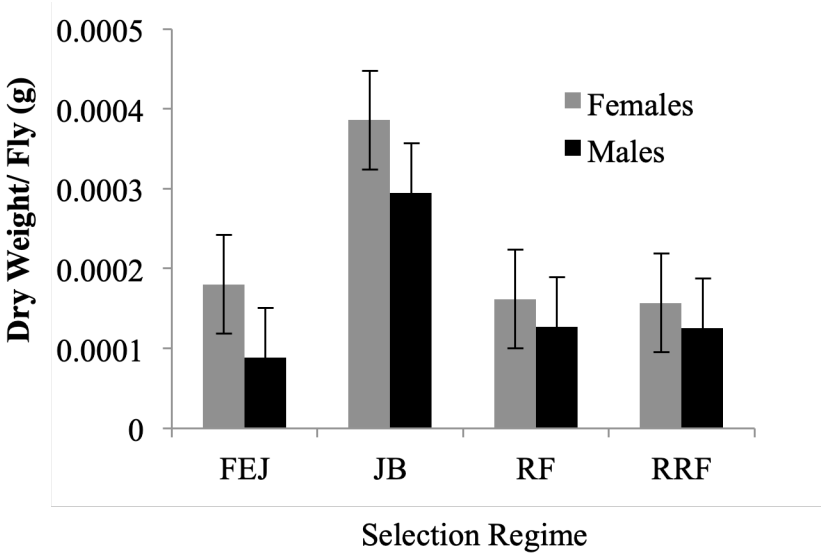


Fig 3.4 Mean dry weights at eclosion of FEJ, JB, RF, and RRF males and females, averaged across four replicate populations, after 40 generations of RRF and 20 generations of RF selection. Error bars are 95% confidence intervals around the means.

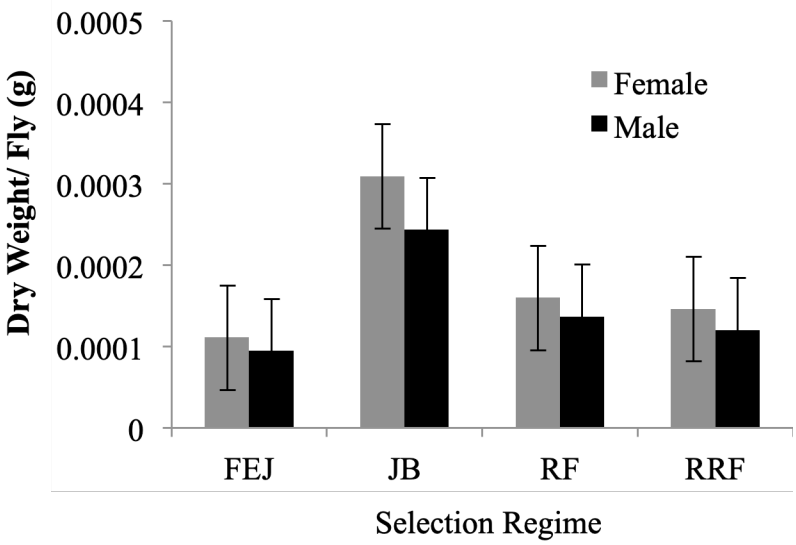


Fig 3.5 Mean dry weights at eclosion of FEJ, JB, RF, and RRF males and females, averaged across four replicate populations, after 60 generations of RRF and 35 generations of RF selection. Error bars are 95% confidence intervals around the means.

Chapter 4:

Estimation of Male Toxicity and Female Resistance in FEJ, JB, RF and RRF

Introduction

Having established that the RF and RRF populations have responded to relaxed selection for rapid development, I next explored the effects of differences in time of reproduction between these populations on inter-locus sexual conflict related traits, and these studies are discussed in this chapter.

The degree of inter-locus sexual conflict can be assessed either by investigating the post mating effect males have on females or by assessing the investment males make into such manipulations. From the male perspective, assessment of the level of courtship (Partridge and Fowler 1990), mating rate (for example; Rice 1996, Wigby and Chapman 2004, Wensing et al 2017) and quality of ejaculate, including sperm and seminal fluid proteins (Linklater et al 2007, Nandy et al 2013a), can be made. These traits however, are difficult to assay. It is easier to examine the response in female fecundity or life-span, after controlled exposure to mates, as both these fitness related traits are implicated in the female post-mating response and are manifestations of inter-locus sexual conflict in *D. melanogaster* (Partridge et al 1986).

Based on the results reported in Chapter 2, there is sufficient reason to expect differences in breeding duration to affect levels of inter-male competition for fertilization of eggs between FEJs and JBs. Since RF and RRF selection regimes differ only in the time available for breeding, exploring inter-locus sexual conflict in these populations can reveal if selection for early reproduction could have contributed towards reduction in inter-locus sexual conflict in the FEJs. If so, differences in levels of sexual conflict can also be expected between the RFs and RRFs, despite their similar body sizes.

Furthermore, RFs and RRFs have responded to relaxation of selection for rapid development with an increase in pre-adult development time and body size at eclosion (Chapter 3). Since FEJ, JB, RF and RRF exhibit a range of different body sizes among them and have shared common ancestry with a four-fold replication within each type, they form a unique system with which to conduct comprehensive and sufficiently conclusive studies to establish the relationships between adult body sizes and inter-locus sexual conflict, adult body size being a trait known to be correlated with sexual selection and conflict (Partridge et al 1987 a, b, Pitnick 1991, Pitnick and Garcia-Gonzales 2002, Mital 2013). I expect body size to be an important explanatory variable for studies reported in this chapter with sexual conflict levels predicted to be different between FEJs and RRFs, and between JBs and RFs given the differences in their body size, despite similar breeding durations.

Since inter-locus sexual conflict is a product of male-female interaction the effects of such interactions (for instance, type of ejaculate and the female physiology) need to be specifically addressed. This may be done by exposing all males to a common female type, or vice-versa. However, since this is a co-evolutionary process, information on the outcome of interactions between males and females from the same population may be missed if common partners are used to assess the effects of different male and female types.

I chose a fully factorial experimental design where every female type was exposed to every male type to investigate the effects of male type, female type, as well as co-evolutionary outcomes of male-female interactions, via statistical interaction effects between male type and female type.

One of the first changes that occur in a female after mating is an increase in egg output (Wolfner et al 1997, 2009 and references therein). This is mediated by an accessory gland protein, Ovulin, also called Acp26A (Chen et al 1988, Herndon and Wolfner 1995, Heifetz et al 2000), and the sex peptide (SP), also called Acp70A (Chapman et al 2003, Lui and Kubli 2003). The sex peptide has also been implicated in mating costs to females in terms of survivorship (Wigby and Chapman 2005). Assuming a limit on the total number of eggs produced by a female in her lifetime, a higher egg lay during the time that sperm from the most recent mating are still stored in the female, coupled with the manipulation of female behaviour to prevent re-mating (Chen et al 1988), ensures that the male sires a higher proportion of the progeny produced by his mate, than would have been the case otherwise. This is a clear fitness advantage for the male but may not be in the evolutionary interest of the female (Chapman et al 2003, Anrqvist and Rowe 2013). Therefore, to obtain a complete picture, the effect of mating with a male on the life-time reproductive success of the female needs to be assessed.

In the experiments described here, the effects of mating with males from different selection regimes ont he mortality rate and fecundity of females were checked. Cumulative mortality was recorded over fourteen days from the set-up (flies were a day old at the time of set-up), whereas fecundity was checked from day one to five after set-up only. Since FEJ adult life-span is effectively about four days, four days was a reasonable time period over which to estimate any differences between the populations with respect to their life-time reproductive success, as affected by the mate provided to them. Cumulative mortality assessment was restricted to the duration of fourteen days from the set-up, since this duration is sufficient to assess any differences in male effects on female mortality (Mital 2013), due to the high intrinsic

mortality of the FEJ females. Although the life-span for each female was also recorded, analysis was done only on data for the first 14 days after set-up.

Additionally, the dry weight of adults at the time of assay set-up was also recorded as the weight of flies is known to change in the first few days after eclosion (Djawdan et al 1996), and I wished to capture the body size differences between the different types of flies, as they were at the start of the assay. I also limited the exposure to males to only 50 hours, to closely resemble the conditions from a previous assay (Mital 2013), as well as limit the time that males were allowed to interact with (and possibly harass) females, as this is known to have an effect on female survival (Partridge and Fowler 1990, Friberg and Arnqvist 2003). The short exposure also ensured that there were unlikely to be major differences in the number of matings achieved by the males, and the results are, therefore, likely to reflect the effects of minimum possible exposure to males (Mital 2013).

The above measures were taken to approximate the assay conditions as closely as possible to those that the populations are adapted, to allow meaningful inferences of the results, relevant to the specific life-history of the populations under study (Sgrò et al 2000, Rice et al 2006, Long et al 2010). Work done earlier on a set of populations that belong to the same lineage of *D. melanogaster* as the JB showed that selection for late life reproduction can significantly affect traits related to male competitive ability (Service 1993, Service and Fales 1994), as well as their ability to induce a high fecundity response soon after mating (Service and Vossbrink 1996), in an age dependent manner.

Methods

Male Toxicity Assay- Post Mating Female Mortality

Two assays of male toxicity effects were conducted, one after 17 and 36 generations of RF and RRF selection, respectively, and the other after 36 and 63 generations of RF and RRF selection, respectively. As each population had a different generation time, it was not possible to synchronize the experiments to the same passage of 'evolutionary' time for the four types of populations. At a time, populations representing all four selection regimes in one block were assayed simultaneously. These assays were also conducted close to the development time experiments reported in the previous chapter, in order to allow easy inference with respect to the direct response to relaxed selection in RF and RRF populations.

From each standardized population (as described in Chapter 1), cultures were initiated with each population staggered in a manner such that adults emerged together. These development time estimates were based on our observations during their regular maintenance, or on the most recent development time experiments. Freshly eclosing adults were collected every six hours to ensure virginity and placed in vials, with others of the same sex, after segregation by sex over light carbon dioxide anesthesia. Once the required number of males and females had been collected from each population, crosses were set up after ~12 hours to reflect all possible combinations of male and female types (selection regime). For each combination, 30 vials were set up with single pairs in each. Fecundity was checked on the first four days. After 50 hours of housing, the males were removed without anesthetization (using the vial flipping technique). After four days of housing for fecundity, 20 vials from each treatment were retained to estimate mortality once every day, providing fresh food to the females every alternate day until all

females died. I calculated cumulative mortality over 14 days for each treatment, from the set-up day.

Male Toxicity Assay- Fecundity over Four Days

Over the first four days of this assay, females (with or without male partners) were kept in vials with a very thin layer of banana-jaggery food medium (<1 mL) just enough the cover the surface of the vial. The food was poured very carefully in order to ensure the walls of the vial were clean as flies would tend to lay eggs on vertical food surfaces, such as food on walls, making it difficult to count. Since eggs begin to hatch around 24 hours after egg lay, females were moved to fresh egg laying vials each day for the first four days. These measures facilitated easy and accurate estimation of egg number in each vial. Fecundity over the first two days was assayed in the presence of males, the second two days in the absence of males. Eggs in each vial were counted and under a microscope, and the numbers recorded, immediately after females were transferred to a fresh vial. Average fecundity of the 30 females from each population was used as the response variable.

Dry weight

At the time of setting up of the 16 male-female combinations, a set of 25 flies was saved and stored at -20^{o}C to be assayed for dry weight later on. Although a set of dry weight estimates was taken close to these set of experiments from the same selected populations (Chapter 3), weight of the flies changes for a few hours and days after eclosion as reproductive maturation continues

and the excreta from their metamorphosis is discarded. Therefore, this assay was done to have an accurate estimate of the differences in their body mass at the time of the male toxicity assay. After the fecundity experiment was completed, five flies each were setup in glass vials, with five replicates per sex, per population for drying and subsequent measurement (see methods section in Chapter 3).

Statistical Analyses

All statistical analyses were done on STATISTICA^{TM} using Windows Release 5.0B (Statsoft Inc. 1995). Mixed-model analysis of variance (ANOVA) was performed with male type and female type (type= selection regime) as fixed factors, and block as a random factor. Since mortality data were fractional, ANOVA was also performed on the arcsine square root transformed data set to see if treatments showed differences in the two analyses for significance. Tukey's HSD test was used for comparing individual means and calculating all confidence intervals for post-hoc analyses.

Results

Post Mating Female Mortality

In both the male toxicity assays, a significant main effect of both male type and female type was seen, with JB males causing highest induced mortality in females, while FEJ females had the highest mortality within fourteen days from set-up (Tables 4.1, 4.2, Figs, 4.2, 4.4). Figures 4.1

and 4.3 show that FEJ female mortality after mating was consistently high, irrespective of male type. The interaction of male and female type was significant only in the second assay, showing that FEJ female mortality when mated with FEJ males was low, but was high when mated with males from the other three types of populations (Fig 4.3). It appears from these results that the intrinsically high mortality of FEJ females (either due to mating stress or otherwise) is resulting in a significant main effect of female type.

Neither mortality induced in females by RF and RRF males (Figs 4.2 A, 4.4 A), nor RF and RRF female susceptibility to male toxicity (Figs 4.2.B, 4.4 B) significantly differed from each other. Main effects of RF and RRF males and females were both intermediate between main effects of FEJ and JB males and females, respectively, and body size differences between the populations could explain these results (Figs 4.11, 4.12). Unlike earlier work, a disproportionate increase in female susceptibility (seen earlier in FEJ females, Mital 2013), over and above body size differences, was not seen in any population in this assay. All arcsine square root transformed data showed the same pattern of significance as untransformed data.

Fecundity over Four Days

Fecundity of females was examined over the first four days after the set-up of the male toxicity assay, and the results again revealed a significant main effect of both female type and male type (Figs 4.5, 4.7, Tables 4.3, 4.4). Fecundity seemed to be directly related to female body size, with JBs showing maximum, FEJs minimum and RFs and RRFs intermediate fecundities (Figs 4.11, 4.12). The RF and RRF populations again did not differ significantly from each other in fecundity. A significant main effect of male type on female fecundity was seen. RFs seemed to

induce maximum egg lay, although the absolute difference in egg output was not more than two eggs (Figs 4.5 B, 4.7 B). In the first assay there was no significant interaction of male type-by-female type (Table 4.3), whereas in the second assay a significant male type-by-female type interaction was seen (Table 4.4), showing RF males inducing greatest egg lay in RF females in the second assay.

To examine the effect of male presence on female egg output, the fecundity results of the first two days were compared with those from the second two days, after the male was removed. A significant interaction effect of male presence-by-female type was seen in both assays (Tables 4.5, 4.6), indicating that different female types responded differently to male presence. JB females tended to lay significantly more eggs in the presence of males (Figs 4.9, 4.10). In the first assay, FEJ, RF and RRF females did not differ in fecundity regardless of male presence or absence, whereas in the second assay FEJ females laid significantly more eggs in the absence than in the presence of males. The significant main effect of female type was retained, as seen in tables 4.5 and 4.6.

Dry Weight

Both dry weight assays revealed a significant main effect of selection, sex and selection-by-sex interaction (Tables 4.7, 4.8, Figs 4.11, 4.12) with the male-female body size difference most pronounced in the JBs, least in the FEJ and similar between the RFs and RRFs. The crucial result here is, again, the lack of any significant difference between the RF and the RRF body sizes. Since these flies were weighed when they were about a day old, compared to freshly eclosed

flies (Chapter 3), some amount of weight was likely shed as the flies became sexually mature, and sex-specific differences might, therefore, have become more pronounced.

Discussion

The post mating response of females can be used to assess the level of inter-locus sexual conflict as it is indicative of both the effect of different types of males on female fitness components after mating, as well as the extent of resistance that different females may mount against such manipulations by males. Flies from the four different types of selection regimes were used, with two of these selected for early reproduction (about three days of adult life: FEJ and RRF) and two for later reproduction (about seven days and eleven days of adult life: RF and JB). By exposing each of the four different types of females to each of the four different types of males, I attempted to distinguish between male manipulation as well as female defense, as readouts for inter-locus sexual conflict. The focus of the work presented in this chapter was to assess if differences in breeding duration mediate changes in inter-locus sexual conflict levels.

Post Mating Female Mortality

The mortality assay results show female type having a significant effect with the smallest females showing maximum mortality in the 14 day period that was examined. There was also a significant effect of male type, with JB males causing most harm, and the pattern suggesting that this effect of increased mortality of mates could be related to male body size. The lack of a significant male type-by-female type interaction in the first assay suggests that the primary

explanatory factor for both male ability to induce harm and female resistance to that harm is the selection regime the respective flies come from. Although this interaction was significant in the second assay, the trend was not starkly different, with FEJ females still showing high mortality with JB, RRF and RF males, which tended to induce greater female mortality, on average, and low mortality only with FEJ males that tended to induce lower female mortality, on average. Similarly, FEJ female mortality was higher than JBs, even when averaged across mate identity, while RF and RRF females showed greater average mortality than JB females and slightly less average mortality than FEJ females. Since these patterns correspond well to the body size difference of flies from the four selection regimes, and larger body size of males is known to contribute towards increased harm to females upon mating in *D. melanogaster* (Pitnick and Garcia-Gonzales 2002, Wigby et al 2016), which was also demonstrated earlier in the FEJ and JB populations (Mital 2013), body size differences may explain these results.

A similar assay was conducted earlier in this lab with size control flies for FEJ (in JB genetic background) specifically to investigate the effects of body size on female post mating mortality (Mital 2013). Results from that assay revealed a significant interaction between male type and female type, with FEJ females showing high susceptibility to JB males, while the size control JB females (as small as the FEJ females) did not show a similarly high susceptibility to the large JB males, indicating that post mating female mortality was governed more by genetic background than by body size. The protocols used in both assays were almost identical, with females being exposed to males for only 50 hours, and cumulative mortality of singly housed females recorded over 14 days, and therefore, can be directly compared. If differences in inter-locus sexual conflict were indeed driven largely by differences in selection for time of reproduction, we should have observed FEJ and RRF females with JB males to have a

disproportionate increase in mortality rate. The results presented here implicate only selection for rapid development (and possibly changes correlated with it, such as adult body size) in affecting inter-locus sexual conflict related traits. Therefore, the differences in time of reproduction between FEJ and JB populations do not appear to have given rise to the genetic architecture that confers high tolerance to smaller JB females. In the current assay RF and RRF females showed higher mortality than JB females, and RF and RRF males also induced greater mortality in FEJ females, corresponding with their body size differences. In this experiment, however, it is not possible to tease apart the effects of body size similarity between the RFs and RRFs, on the one hand, and FEJs on the other, and closer degree of relatedness of the RFs and RRFs to the FEJs, compared to the JBs.

I would like to point out some minor differences between the protocols of the earlier assay (Mital 2013) and the current assays presented in this chapter. In the earlier assay, only 10 females were set up to check for mortality compared to 20 females in this assay, which provides this set of assays with a larger sample size for analysis. Additionally, since fecundity was also checked for the first four days in the current assays, only females that survived the first four days were set up for mortality such that at least 20 females were available for every male type-by-female type combination at the start of the mortality checks. This could result in selection of females that were slightly better at surviving post-mating stress, and therefore, would yield overall lower cumulative mortality values. The cumulative mortality values seen in the current assays are indeed slightly lower than those seen earlier (Mital 2013).

The strongest evidence, however, that breeding ecology does not seem to give rise to any major differences in either male mate harming ability, or female resistance to post mating manipulations, comes from the comparison of the flies from the RF and RRF regimes. In none of

the results described above is there a significant difference between the RF and RRF males in eliciting either increased fecundity (Figs.4.5- 4.8) or increased mortality (Figs.4.1-4.4), countering the hypothesis proposed in Chapter 2 of this thesis. This strongly suggests that the duration of adult life until reproduction, per se, does not seem to have any major effect on the levels of inter-locus sexual conflict in these populations.

Fecundity over Four Days

A significant main effect of female type in both fecundity assays revealed a significant difference between the selection regimes, with the FEJ having the least and JB the highest fecundity (Figs 4.5, 4.7). Evidence that fecundity in female fruit flies is highly correlated with body size is extensive (Mueller 1985, Chippindale et al 1994, Prasad and Joshi 2003) and the results are consistent with this expectation, as the fecundity pattern follows the body size pattern of the different female types very closely (Figs 4.11, 4.22).

Other laboratories have reported male type having an effect on female fecundity, especially soon after copulation (Pitnick 1991, Pitnick and Garcia-Gonzales 2002, Schultzhaus and Carney 2017). Male body size has been implicated in the degree of post-mating response elicited by males in females (Pitnick 1991, Pitnick and Garcia-Gonzales 2002, Friberg and Arnqvist 2003), including egg output. The current results seem to contradict these studies as RF males appeared to increase female fecundity significantly more than other male types (Figs 4.6 B, 4.8 B), although the absolute differences in fecundity were not high. Moreover, the trends for the remaining three selection regimes were not consistent from the first assay to the next, only the females mated with RF males showed maximum egg output in both assays. FEJ males

seemed to elicit a high fecundity response in the first assay (Fig. 4.6 B) but elicited the least fecundity in the second assay (Fig.4.8 B), while JB males showed the opposite trend.

I believe that, despite the statistically significant differences in main effect of males, these are unlikely to be biologically significant for the purpose of this investigation, as the absolute values of induced fecundity were not very different between different male types, in either assay, with the pattern also being inconsistent across assays. Given the sampling of eggs inherent in egg collection during routine population maintenance in these selection regimes, very small differences in fecundity may not translate into consistent fitness differences among individuals. It is possible that the high degrees of freedom offered by the large number of means used in the comparisons (Tables 4.3, 4.4) could have resulted in such minor differences turning out to be statistically significant.

Long et al (2010) also report the effects of mating soon before the relevant oviposition window, assessed in the LH_m *D. melanogaster* populations that are maintained on a two-week discrete generation cycle. They found the effects of body size of males to have little effect on the fecundity of females within the two day period of egg lay after mating, as did Pitnick and Garcia-Gonzales (2002), who found only long term effects of male body size on female fecundity and survivorship. These reports are supported by the results presented here and I speculate that high induced fecundity by any male type within a two/three day period after mating likely renders the Acps implicated in this manipulation (Acp26A and Acp70A) beneficial to FEJ females as they are required to lay eggs within that time period and do not benefit from a long subsequent adult life. Therefore, these Acps might even be considered to no longer be mediating inter-locus sexual conflict between males and females as they would confer a fitness advantage to both sexes in the FEJ populations.

The design of this experiment also allowed the investigation of non-mating effect of male presence on female egg lay. Since there are non-mating costs to females in terms of longevity, via harassment due to courtship (Partridge and Fowler 1990, Friberg and Arnqvist 2003), I wanted to investigate if persistent male courtship could interfere with female oviposition. The results here demonstrate no significant effect of male presence, when the first two days were compared with the last two days of egg lay (Figs 4.9, 4.10). A significant interaction effect of female type with male presence was seen in both assays, with JB females laying more eggs in the presence of males whereas FEJ females laid more eggs in the absence of males. RFs and RRFs did not show any differences in egg lay, based on male presence. One possibility is that these data are confounded with age and day of recording, as the male presence treatment was sequentially conducted. Since I have looked at egg lay within the first five days of adult life, wherein fecundity is typically high, this effect may not be large. Differential effect of male type, with FEJ females being more susceptible to harassment than JB females, could also give rise to these results. FEJ females do not appear to actively avoid chasing and sit benignly while being courted (FEJ males also exhibit much less chasing), whereas JB flies, both males and females, are highly active during courtship. It is not clear what might cause JB females to lay more eggs in the presence of males than in their absence, when lack of viable sperm is unlikely to be a causal factor. JB females are possibly more dependent on a fresh supply of sperm for large egg output, at least early in life. Since results from Chapter 2 have shown low mating rates for JBs closer to egg laying day (day 21), they potentially start using stored sperm preferentially only later. I do not present any results to corroborate these claims, and further investigation into the effects of male presence on FEJ and JB females is required. It is clear, however, that male presence can significantly reduce FEJ female fitness, but not JB female fitness.

Overall, the post mating response of females in terms of increased egg output was not affected by the type of male (based either on selection history or body size), indicating that there is little difference between the males of different selection regimes in their ability to increase female egg lay. Thus, females from these populations do not experience a fitness loss in terms of fecundity upon exposure to males for 50 hours, which covers the life-time egg output for FEJ and RRF females. Possibly, development time is the only driving force in bringing about the changes in traits traditionally associated with inter-locus sexual conflict in this system.

In conclusion, these results support the hypothesis that the ability of males to elicit a post mating response in females, in terms of mortality, seems to be primarily a function of selection for rapid development per se, or due to the strong correlated response of body size. The degree of mate manipulation by males is known to be positively correlated with body size differences in *D. melanogaster* (Pitnick and Garcia-Gonzalez 2002, Mital 2013, Wigby et al 2016), and female resistance to post mating harm probably changes via a co-evolutionary process. Female fecundity, immediately after mating, also seems to be primarily a function of the selection regime the female belongs to, and is not markedly affected by male type, a pattern that may also be explained by body size differences, consistent with earlier results from the same set of populations in this laboratory (Prasad 2004).

Tables

Table 4.1 Summary of the ANOVA results of cumulative mortality over 14 days after exposure to males for 50 hours, after generations 36 of RRF and 17 of RF selection, with male and female type as fixed factors, and block as a random factor. This design does not allow testing of random factors and interactions for significance and they have been omitted for brevity.

Effect	**df**	**MS**	***F***	***P***
Male Type	3	0.0419	5.122	0.024
Female Type	3	0.1505	6.924	0.010
Male × Female Type	9	0.0106	1.579	0.171

Table 4.2 Summary of the ANOVA results of cumulative mortality over 14 days after exposure to males for 50 hours, after generations 63 of RRF and 36 of RF selection, with male and female type as fixed factors, and block as a random factor. This design does not allow testing of random factors and interactions for significance and these have been omitted for brevity.

Effect	**df**	**MS**	***F***	***P***
Male Type	3	0.0797	21.797	<0.001
Female Type	3	0.0520	12.553	0.001
Male × Female Type	9	0.0103	2.439	0.035

Table 4.3 Summary of the ANOVA results of daily fecundity in the first four-five days including exposure to males for 50 hours, after generations 36 of RRF and 17 of RF selection, with male type and female type as fixed factors, and block as a random factor. This design does not allow

testing of random factors and interactions for significance and these have been omitted for brevity.

Effect	**df**	**MS**	***F***	***P***
Male Type	3	13.5252	3.952	0.047
Female Type	3	1487.952	111.869	<0.001
Male × Female Type	9	6.9690	2.208	0.054

Table 4.4 Summary of the ANOVA results of daily fecundity in the first four-five days including exposure to males for 50 hours, after generations 63 of RRF and 36 of RF selection, with male and female type as fixed factors, and block as a random factor. This design does not allow testing of random factors and interactions for significance and these have been omitted for brevity.

Effect	**df**	**MS**	***F***	***P***
Male Type	3	19.4721	5.456	0.020
Female Type	3	800.36111	94.479	<0.001
Male × Female Type	9	10.2847	5.670	<0.001

Table 4.5 Summary of results of ANOVA performed on daily female fecundity with female type and male presence as fixed factors, and block as a random factor, conducted after generations 36 of RRF and 17 of RF selection. This design does not allow testing of random factors and interactions for significance and these have been omitted for brevity.

Effect	df	MS	*F*	*P*
Female Type	3	2953.711	109.945	<0.001
Male Presence	1	178.605	1.939	0.257
Female Type × Male Pres.	3	248.7527	12.920	0.001

Table 4.6 Summary of results of ANOVA performed on daily female fecundity with male type, female type and male presence as fixed factors, and block as a random factor, conducted after generations 63 of RRF and 36 of RF selection. This design does not allow testing of random factors and interactions for significance and these have been omitted for brevity.

Effect	df	MS	*F*	*P*
Female Type	3	1596.963	97.152	<0.001
Male Type	3	37.1197	4.989	0.026
Male Presence	1	0.4626	0.190	0.691
Female Type × Male Pres.	3	112.7262	60.722	<0.001
Male Type × Male Pres	3	45.2740	12.253	0.001
Female Type × Male Type × Male Pres.	9	9.5967	5.982	<0.001

Table 4.7 Summary of ANOVA on dry weight of flies at the time of male toxicity assay set-up after 36 generations of RRF and 17 generations of RF selection, with selection and sex as fixed factors, and block as a random factor. This design does not allow testing of random factors and interactions for significance and these have been omitted for brevity.

Effect	df	MS	*F*	*P*
Selection	3	558.9001	106.633	<0.001
Sex	1	544.775	119.946	0.001
Selection × Sex	3	26.9651	8.371	0.005

Table 4.8 Summary of ANOVA on dry weight of flies at the time of male toxicity assay set-up after 63 generations of RRF and 36 generations of RF selection, with selection and sex as fixed factors, and block as a random factor. This design does not allow testing of random factors and interactions for significance and these have been omitted for brevity.

Effect	df	MS	*F*	*P*
Selection	3	$5.0871 \times 10^{\wedge -8}$	52.968	<0.001
Sex	1	$1.2163 \times 10^{\wedge -8}$	25.523	0.014
Selection × Sex	3	$2.8052 \times 10^{\wedge -9}$	11.688	0.001

Figures

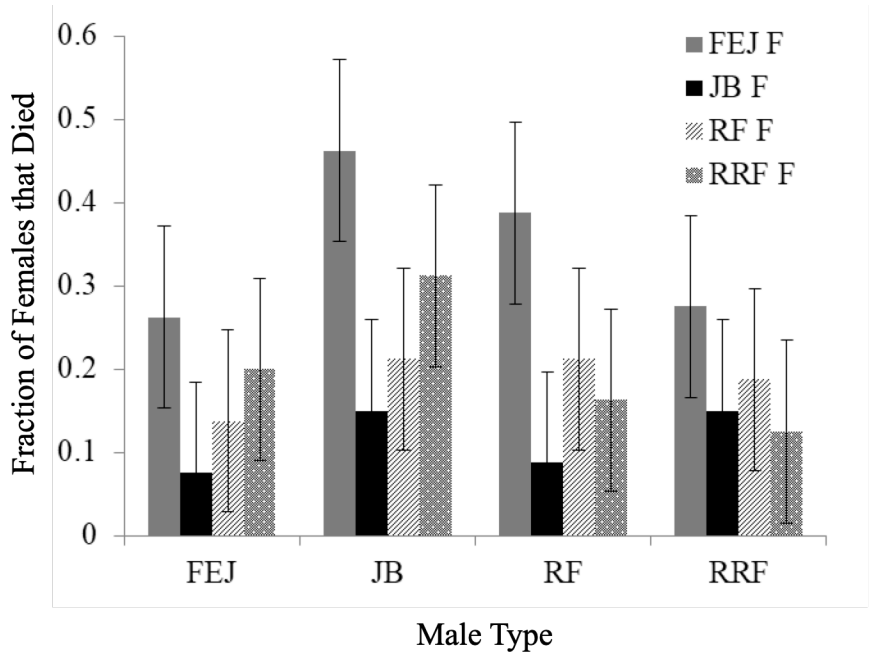


Fig 4.1 Mean cumulative female mortality over 14 days after 50 hours of exposure to males, after generations 36 of RRF and 17 of RF selection, averaged across four replicate populations. Error bars are 95% confidence intervals around the means.

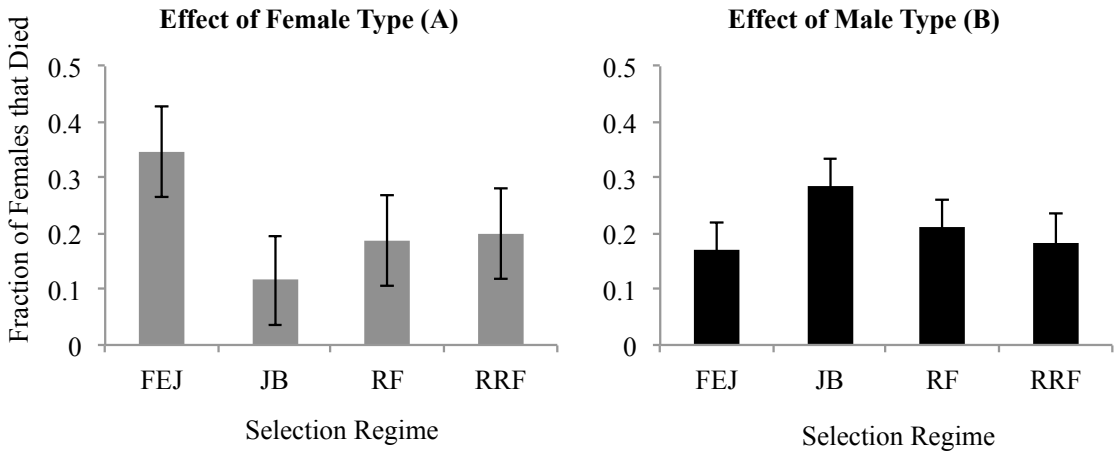


Fig 4.2 Mean cumulative mortality of females, averaged across four replicate populations, over 14 days with 50 hours of exposure to males, after 36 generations of RRF and 17 generations of RF selection, reflecting A) the main effect of female type, and B) the main effect of male type. Error bars are 95% confidence intervals around the means.

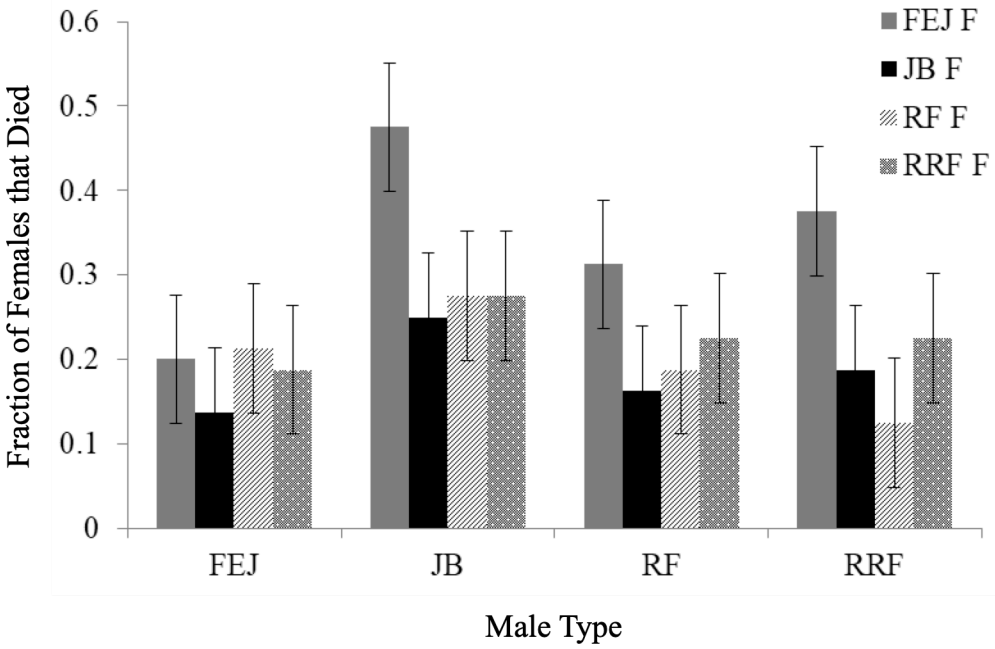


Fig 4.3 Mean cumulative female mortality over 14 days after 50 hours of exposure to males, after generations 63 of RRF and 36 of RF selection, averaged across four replicate populations. Error bars are 95% confidence intervals around the means.

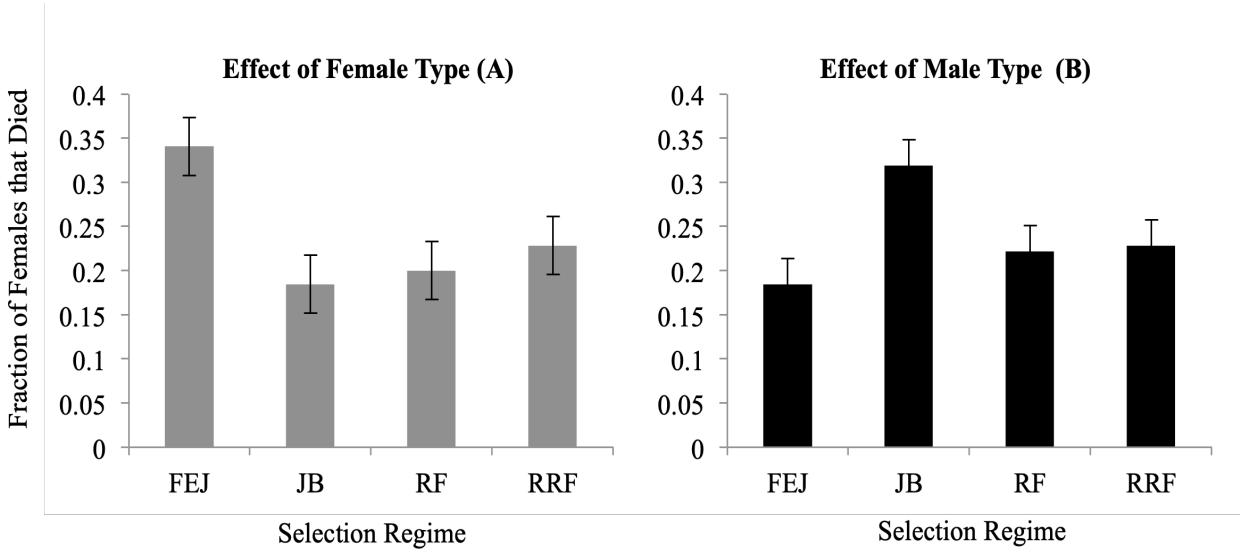


Fig 4.4 Mean cumulative mortality of females, averaged across four replicate populations, over 14 days with 50 hours of exposure to males, after 63 generations of RRF and 36 generations of RF selection, reflecting A) the main effect of female type, and B) the main effect of male type. Error bars are 95% confidence intervals around the means.

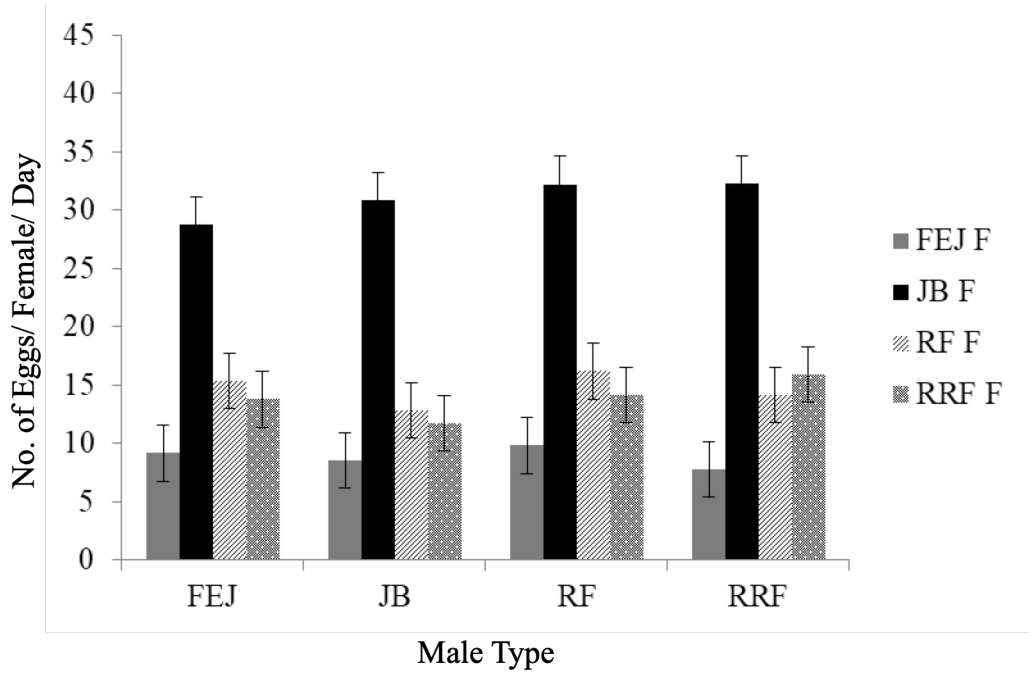


Fig 4.5 Mean fecundity over four days, including exposure of females to males for 50 hours, after generations 36 of RRF and 17 of RF selection, averaged over four replicate populations. Error bars are 95% confidence intervals around the means.

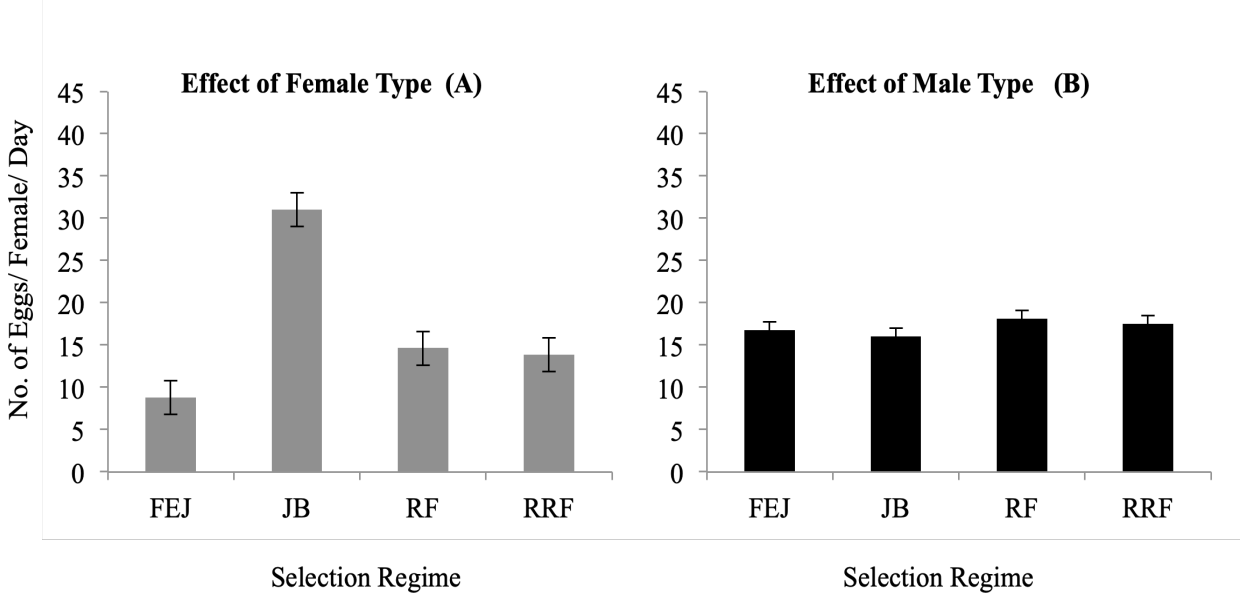


Fig 4.6 Mean fecundity of females, averaged across four replicate populations, in the first four-five days of life including 50 hours of exposure to males after 36 generations of RRF and 17 generations of RF selection, reflecting A) the main effect of female type, and B) the main effect of male type. Error bars are 95% confidence intervals around the means.

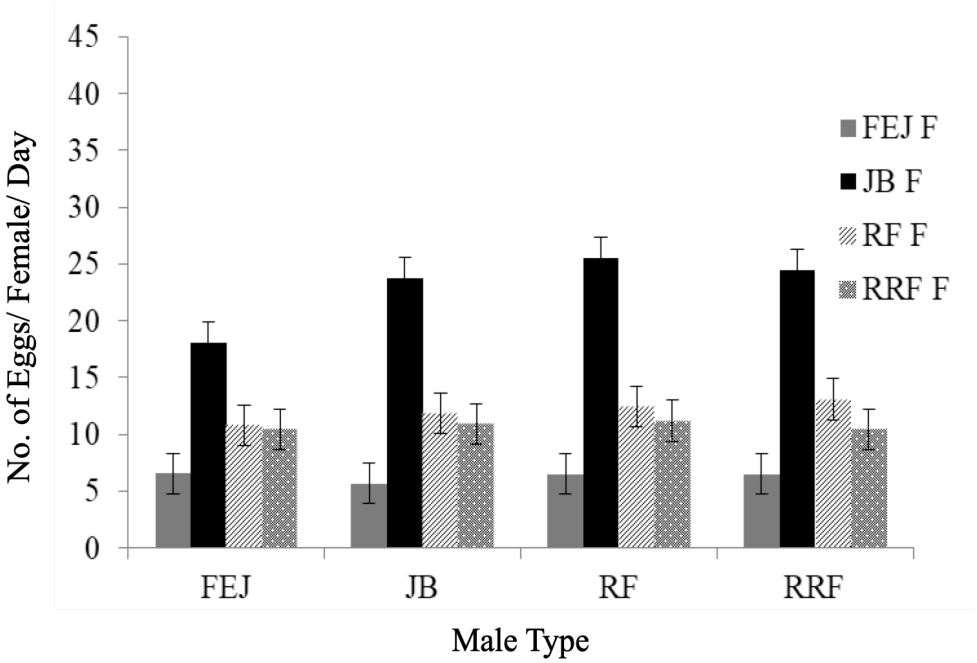


Fig 4.7 Mean fecundity of females, averaged across four replicate populations, in the first four-five days of life including exposure of females to males for 50 hours, after generations 63 of RRF and 36 of RF selection. Error bars are 95% confidence intervals around the means.

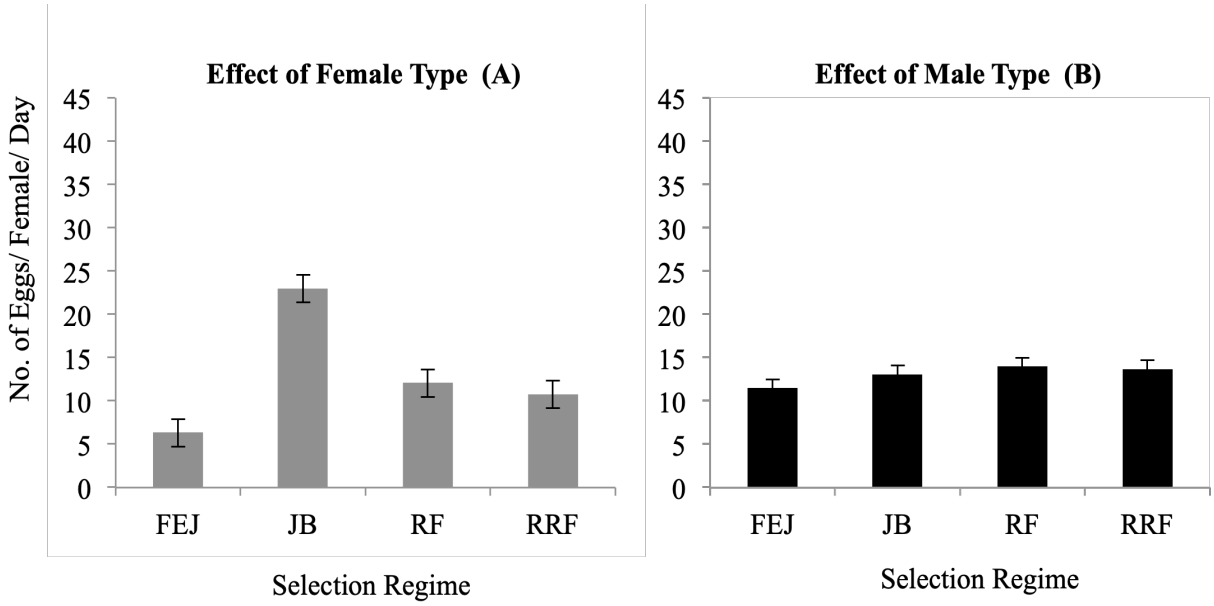


Fig 4.8 Mean fecundity of females, averaged across four replicate populations, in the first four-five days of life, including 50 hours of exposure to males, after 63 generations of RRF and 36 generations of RF selection, reflecting A) the main effect of female type, and B) the main effect of male type. Error bars are 95% confidence intervals around the means.

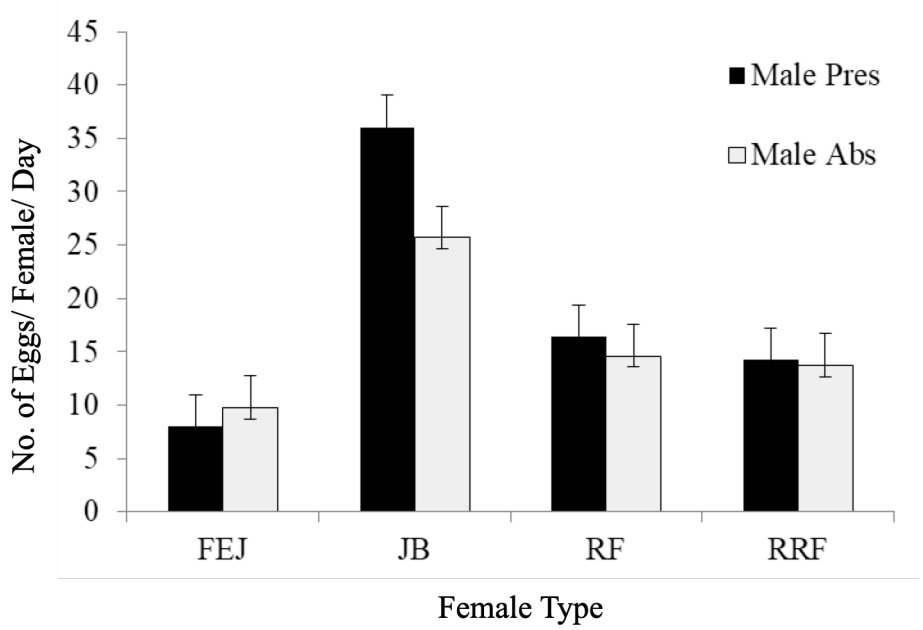


Fig 4.9 Mean fecundity of females during male presence and absence, in the first four-five days of life, after 36 generations of RRF and 17 generations of RF selection, averaged across four replicate populations. Error bars are 95% confidence intervals around the means.

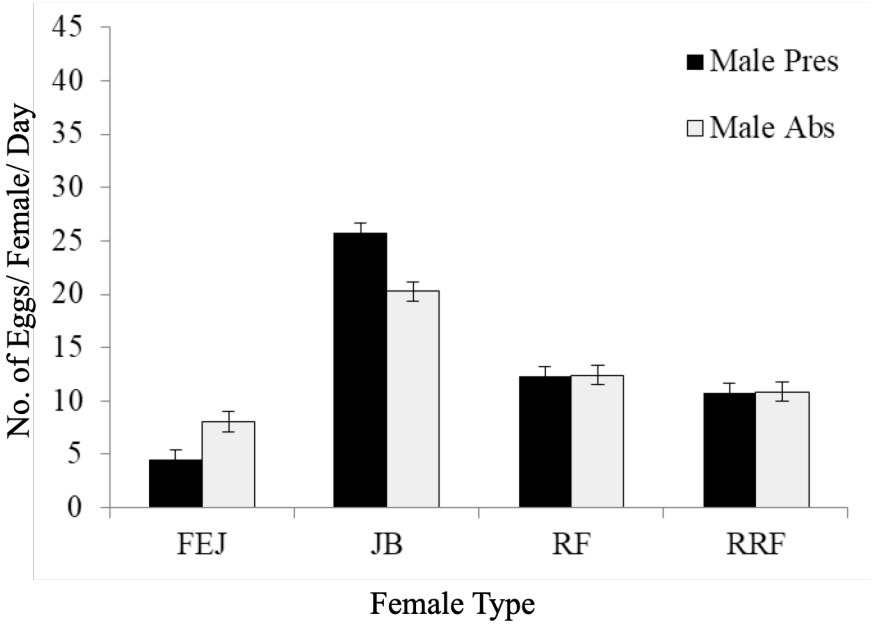


Fig 4.10 Mean fecundity of females during male presence and absence, in the first four-five days of life, after 63 generations of RRF and 36 generations of RF selection, averaged across four replicate populations. Error bars are 95% confidence intervals around the means.

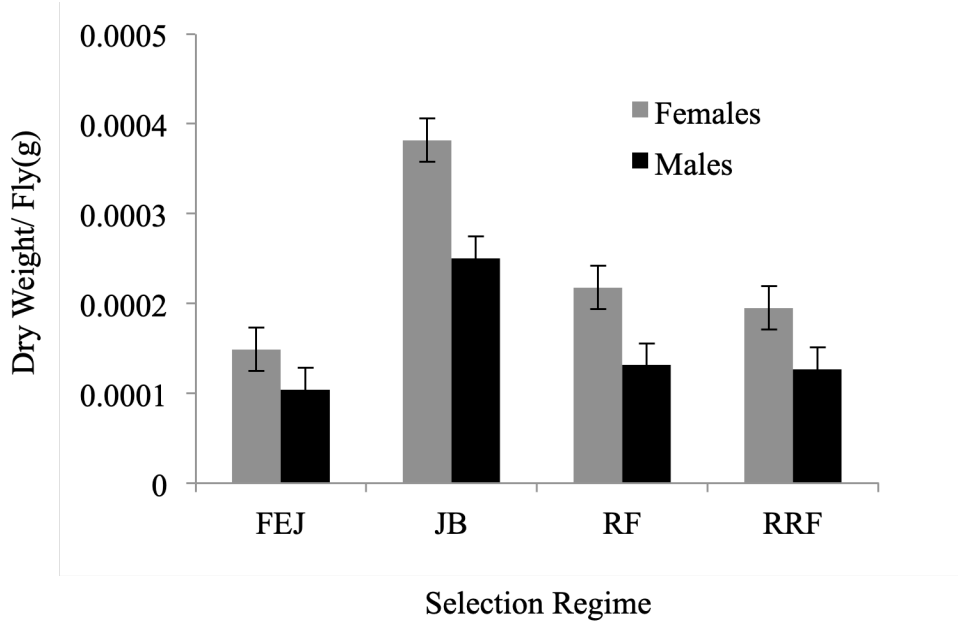


Fig 4.11 Mean dry weights of males and females on day one after eclosion, at the time of male toxicity assay set-up, conducted after generations 36 of RRF and 17 of RF selection, averaged over four replicate populations. Error bars are 95% confidence intervals around the means.

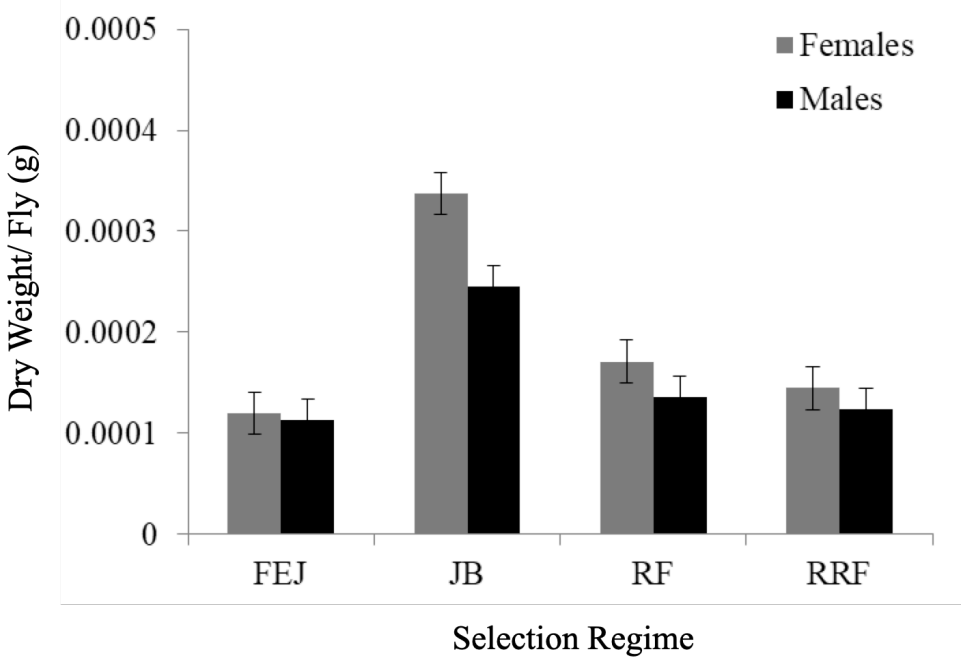


Fig 4.12 Mean dry weights of males and females on day one after eclosion, at the time of male toxicity assay set-up, conducted after generations 63 of RRF and 36 of RF selection, averaged over four replicate populations. Error bars are 95% confidence intervals around the means.

Chapter 5:

Testis and Accessory Gland Size in

FEJ, JB, RF and RRF &

Copulation Duration and Maturation Time of

RF and RRF Females

Introduction

Under conditions of high competition among males for reproductive success, there are various stages in which a male may express traits that give him a reproductive fitness advantage over other males, starting from successful courtship to post-copulatory manipulation of female physiology and behaviour. Depending on the kind of ecology and life-history of the population under study, the specific traits mediating these competitive strategies may change (Wedell et al 2006), as also exemplified in the studies conducted on the B and O *D. melanogaster* populations selected for different ages of reproduction (Service 1993, Service and Fales, 1994, Service and Vossbrink 1996), where the male strategy for manipulation of female behaviour varied in an age specific manner.

In the preceding chapter, an indirect measure of the ability of males to manipulate the post mating response of females in terms of fecundity and survivorship was established, and the different selection regimes were compared in this regard. In this chapter, a direct measure of male investment in reproduction is examined. The ability of a male to evoke the post mating response is determined by the quality and quantity of accessory gland proteins (produced in the accessory gland) that it transfers to the female (Chapman et al 1995), while sperm competition (sperm defense and offence abilities) is largely affected by sperm length and its co-evolution with seminal receptacle size in the female (Pitnick andMiller 2000).

From the results discussed in Chapter 1, the level of male-male competition in the FEJ ecology has been established to be significantly less than that in the JB ecology, with the FEJ being effectively monogamous. Under such conditions of low levels of mating competition, sperm competition should be significantly reduced. Nandy et al (2013a) showed that both sperm

offense and sperm defense ability evolved to be higher in *D. melanogaster* populations maintained under a male biased sex ratio compared to those maintained under a female biased sex ratio.

Given the above mentioned results, a measure of changes in accessory gland size as well as testis size, which are strongly correlated with male competitive fitness (Bangham et al 2002, Wigby et al 2009), while scaling for body size differences, can directly assess male investment in reproduction related traits in the populations studied by me. Moreover, sperm number and length, both correlated with testes size, are known to be involved in sperm competition in *Drosophila sp.* (Snook 2005). Change in testes size in populations experiencing monogamy, relative to polygamous populations, over and above body size reduction, has also been reported in *D. melanogaster* (Pitnick et al 2001). Wigby et al (2016) found that smaller *D. melanogaster* males(i.e. males reared as larvae under low food conditions) tend to invest more heavily in terms of the quantity of ejaculate per mating than do large males, a plastic response probably facilitating the maximization of competitive success. Moreover, Linklater et al (2007) reported differences in ejaculate depletion patterns over successive matings, demonstrating plasticity in terms of investment per mating, depending on the level of competition and available resource. They also showed that this depletion pattern itself evolved in populations adapted to different in levels of sperm competition. Ejaculate amount has been also linked to copulation duration (Gilchrist and Partridge 2000), and *D. melanogaster* males have also been shown to vary copulation duration based on the perceived level of sperm competition (Bretman et al 2009, Nandy and Prasad 2011). Therefore, body size, as well as sexual selection and inter-locus sexual conflict levels can modulate ejaculate investment.

Here, I explore the possibility of disproportionate changes in testis size and accessory gland size (after Chechi et al 2017) in the FEJ, JB, RF and RRF populations, depending on their specific requirements based on the differences in their adult age at effective reproduction. Additionally, constraints imposed by selection for rapid development can limit the absolute size of these organs, while still retaining compensatory investment per mating by FEJ males, if selected for. Finally, to aid faster development in the FEJ, earlier work from this lab has revealed a disproportionate increase in the ring gland size of second and third instar larvae (*J. Bose, A. Joshi, unpubl. data*) possibly allowing quicker moulting, supporting the possibility of altered organ size to body size allometry evolving in these populations.

Assuming that a likely explanation for not seeing differences in terms of inter-locus sexual conflict related traits between RF and RRF is the lack of adequate selection pressure facilitating such a divergence, an investigation into the reproductive behaviour of these populations was called for. I have not investigated the nature of breeding ecology differences with respect to mating and courtship rates in the RFs and RRFs, and RRFs might not be even monogamous like the FEJs, despite a similar breeding time. However, as a preliminary start to these investigations, I have looked at their copulation duration and time till reproductive maturity (time till first mating from eclosion).

Methods

Storing Mated and Virgin Males

Two sets of measurements were done to get an estimate of the reduction in accessory gland and testis size. After collecting same-aged flies from the four different selection regimes, as described in Chapter 1, they were housed as virgins for a day in order to allow sexual maturation to be completed. Fifty single pairs per population were then set up, in individual vials, using carbon dioxide anesthesia, to allow the males to mate. As soon as mating was completed in a vial, the vial was dipped for 20 seconds in liquid nitrogen to flash freeze the flies and halt any changes in physiology and anatomy. Similarly, 50 virgin males from each population were also flash frozen, at the start of the mating treatment setup. Frozen flies were then immediately transferred to -20^{o}C for storage until dissections could be done.

Dissection and Imaging

30 frozen flies were sampled per population for dissections. The fly to be dissected was put on a clean glass slide with its wings clipped off, and photographed in order to measure thorax length and abdomen length for body size estimates. Using a pair of sewing needles mounted on pencils, the fly was held in place by its thorax, and the posterior tip was pressed and pulled, allowing the entire internal male reproductive tract to be pulled out. The testes are easily identifiable as yellow, paired and coiled structures, while accessory glands are paired, white bulbous structures, one along with each testis (Fig. 5.1). All dissections were performed using 1.5 mL of phosphate

buffer solution per fly. The extraneous tissues were removed, and the testes uncoiled, using a pair of very fine, soft-bristled, paint brushes.

The testes and accessory glands were immediately photographed using a Nikon Eclipse E200 microscope with camera attachment. Measurements were performed using the 'perimeter' marking tool in ImageJ software Ver. 1.8.0 for Windows (Rasband NIH, USA) to mark the outline of the structure of interest, and the area projected by the software for the marked region was used for further analysis. The absolute scale in the software was set such that 1.68mm distance was equal to 516 pixels on the photographs. An average of the pair (testes or accessory gland) was taken, and an average of all 30 such readings was used as the mean for each population-by-treatment (virgin/mated) combination. At a time, one block was assayed, with a total of 480 males dissected across all selection regimes per block.

Copulation Duration and Maturation Time

Copulation duration and maturation time in the RF and RRF populations was assayed according to the protocol described in Chapter 2 and the experiment was conducted along with the one reporting the differences between FEJs and JBs (Chapter 2). At the time, RFs and RRFs had undergone 85 and 130 generations of relaxed selection, respectively. The FEJ and JB data is shown here again for the purpose of comparison (Fig. 5.11). This assay was also conducted under both yeasted and un-yeasted conditions, as RFs experience un-yeasted conditions immediately after eclosion, like JB, whereas RRFs experience yeasted conditions after eclosion, like FEJ.

Statistical Analyses

Individual measurements for each male were scaled by the square of its thorax length (Fig 5.1) to assess differences in proportional organ size over and above the large absolute body size differences. Mixed model analysis of variance (ANOVA) was performed on the population means, with block being treated as a random factor, and selection regime and virgin/mated treatment as fixed factors. Mixed model ANOVA was also performed on the log of these ratios. Tukey's HSD test was used for comparing individual means and calculating all confidence intervals for post-hoc analyses. Percentage reduction (after mating) in AG size was also calculated for each population. Analysis was done on both arcsine square root transformed data, and untransformed data, in order to see whether possible departures from normality of the scaled nature of the data gave different results. Data for copulation duration and maturation time in RFs and RRFs were subjected to mixed model ANOVA with selection regime and treatment (yeasted vs non-yeasted) as fixed factors, and block as a random factor. All analyses were carried out on STATISTICA^{TM} using Windows Release 5.0B (Statsoft Inc. 1995).

Results

Testis Size

A significant main effect of selection regime was seen on absolute testis sizes (F=44.511, P<0.001) and when testis sizes were scaled by the respective thorax sizes (F=19.099, P<0.001, Tables 5.1, 5.2, Figs 5.2, 5.3). Absolute testis size of FEJ was the smallest, significantly less than that of JB and RF testis sizes. However, FEJ testis size was found to be proportionally the largest, significantly more than JB testis sizes, while no significant differences were seen

between the RFs and RRFs, either in absolute size or that scaled by thorax size.There was no significant effect of mating status or of testis size-by-mating status interaction in either absolute or scaled testis size, with testis size showing little difference between virgin and once mated males (Table 5.1, 5.2, Fig 5.2. 5.3). The log transformed data showed the same pattern of significance as the untransformed data, reported here.

Accessory Gland (AG) Size

There was a significant main effect of selection regime (F=111.397, P<0.001, F=7.059, P=0.009) and of mating status (F= 231.226, P<0.001, F=172.826, P=<0.001)on AG size (Table 5.3) and on AG size scaled by square of thorax length (Table 5.4), respectively. FEJs had significantly smaller AGs compared to the other three regimes, which were not significantly different from each other (Table 5.3, Fig 5.4). However, JBs showed the smallest proportional AG size, when scaled against squared thorax length (Fig. 5.5). Mated flies had smaller AGs than virgins, significantly so on scaled data as well (Figs 5.4, 5.5),and this difference was similar for the different selection regimes, as indicated by the lack of a significant selection-by-mating status interaction effect (Tables 5.3, 5.4, Figs 5.4, 5.5). The similarity in the reductions of AGs after the first mating is shown in Figure 5.6 (for both absolute reduction and scaled values). No significant effect of selection was revealed here (Table 5.5). The log transformed and arcsine square root transformed data (not shown) revealed the same pattern of significance as the untransformed data reported here.

Copulation Duration and Maturation Time

A significant main effect of selection regime was seen for both maturation time (F= 31.5385, P<0.001) and copulation duration (F=3.8676, P=0.0498) but there was no significant difference between RFs and RRFs in either copulation duration (Fig. 5.11) or maturation time (Fig. 5.12). Furthermore, no significant difference between yeasted or un-yeasted condition was seen (Tables 5.3, 5.4).

Discussion

Three predictions based on theory and experimental evolution studies were tested here. One, testis size is expected to evolve, given differences in the degree of sexual selection between FEJs and JBs, with lower levels of selection resulting in lower investment by males, and possibly a lower sperm requirement (Pitnick et al 2001).

Testis size is correlated with both sperm morphology and number, and these in turn are under strong sexual selection (Pitnick 1996, Pitnick et al 2001, Snook2005), often resulting in rapid divergence in testis size between populations of the same species (Pitnick and Miller 2000) which can play a role in speciation (Manier et al 2013b). Changes in sperm morphology can drive testis size differences (Lüpold et al 2016, Lüpold and Pitnick 2018), thereby playing a role in speciation (Manier et al 2013b). FEJ and JB populations showed indications of incipient reproductive isolation (Ghosh and Joshi 2012), the first step towards speciation, as well as large differences in levels of sexual selection (Chapter 2). My results showed significant differences

between FEJs and JBs in testis size, FEJs having evolved disproportionately larger testes, indicating higher proportional investment in reproduction compared to JBs.

Second, I expected smaller AGs in the FEJs, relative to body size, assuming a potentially lower requirement for manipulative seminal fluid proteins (Satish 2010, Ghosh and Joshi 2012, Mital 2013), as accessory gland proteins are key mediators of inter-locus sexual conflict in *Drosophila sp*. (Wolfner 1997). Surprisingly, although a significant effect of selection was seen on scaled AG size, it was the JBs that showed smaller AG size, proportional to body size, compared to the RFs and RRFs (Fig 5.5).These results indicate that JBs invest proportionately less towards reproduction (in terms of testis size and accessory gland size, compared to FEJ, RF and RRF). In contrast, lower Acp transcription levels, compared to the JBs, have been reported in young males of the FEJ populations (Satish 2010, P Dey and A Joshi, *unpubl. data*).

In terms of absolute organ size, FEJ males have the smallest testes and AGs, as compared to JB, RF and RRF. Thus, the observed reduction in male manipulative ability and female resistance in FEJs (Chapter 3, Mital, 2013) can be explained by the reduction in body size and, consequently, absolute AG size in the FEJs. However, the extremely small body size of the FEJs appears to have driven the evolution of a higher proportional investment in reproduction compared to the JBs, possibly in order to maintain a minimum level of reproductive success. Moreover, given the extremely short effective adult life of the FEJ females (three days), the role of Acps in increasing egg output of females immediately post mating, even at the cost of subsequent lifespan, would likely benefit FEJ females, thus reducing (or eliminating) conflict over those proteins. These two mechanisms are a likely explanation of the overall lower levels of conflict in the FEJ compared to JB, despite the increased proportional investment made by the FEJs in testes and AGs.

The plots of organ size against square of thorax length appeared to be more or less linear (Figs. 5.7-5.10), but regression analyses on data from each selection regime separately showed no clear or strong linear relationship between organ size and square of thorax length for any selection regime (analysis not shown). The pattern seen in figures 5.9 and 5.10 suggested the possibility of an inflection point in the data of AG plotted against squared thorax length. Therefore, I measured the slope of a simple linear regression of AG on squared thorax length for the FEJ, RF and RRF populations, and also for the RF, RRF and JBs, for both virgin and mated flies. A significant difference was seen between these two slopes (F=46.831, P=0.006) with the slope through FEJs being higher than that through JBs, indicating that the relationship between AG size and body size is not consistent across the entire range of the FEJ-JB body size difference.

The above results are consistent with both Linklater et al (2007), who did not find changes in testis and AG size, despite change in ejaculate depletion patters, as well as Chechi et al (2017), who also estimated these parameters in populations previously shown to be divergent in sperm competition related traits (Nandy et al 2013a). Since no significant differences were seen between the RFs and RRFs, selection for rapid development seems to drive changes in the investment towards reproduction, that are not explained by differences in breeding ecology alone.

The third and final prediction was that there might be a smaller reduction in the size of AG post mating, also indicating a lower investment in reproduction related traits by the FEJ, compared to JB (Linklater et al 2007). I found no difference between the selection regimes in terms of AG size reduction after their first mating (Fig 5.6). Additionally, using the RF and RRF populations allowed me to investigate the role of differences in age at reproduction (or breeding

time) on testis and AG sizes, if such selection had led to differences in the level of sexual selection between them (Chapter 2). The lack of any difference between RFs and RRFs seen here is consistent with the results from the previous chapter, indicating no major role of time of reproduction (or operative adult life), by itself, on traits related to sexual selection or inter-locus sexual conflict, at least in these populations.

Despite differences in breeding duration, there is little difference between RFs and RRFs with respect to copulation duration and maturation time. This preliminary investigation also reveals that RF and RRF females are closer in terms of these behaviours to JB females than they are to FEJs, as the only significant differences were between FEJs and the remaining selection regimes (Fig. 5.11, 5.12). The release from selection for rapid development has caused these traits to approach ancestral trait values, at similar rates in the RF and RRF populations, again implicating rapid development and not early reproduction in modulating reproductive behavior. This is also the first indication that perhaps the degree to which the breeding ecologies differ between the RFs and RRFs is insufficient to bring about any significant changes in levels of sexual selection, in the absence of strong selection on development time. Further investigation into mating rates and courtship rates in the relaxed selection populations is required.

Based on the results reported so far, it appears that the hypothesis established in Chapter 2 of this thesis should be rejected. The only evidence suggesting reduction in inter-locus sexual conflict is the smaller absolute AG size in the FEJ populations. Moreover, between RF and RRFs, no differences in AG size were seen. Perhaps, the unique combination of selection for rapid development and early reproduction act synergistically to reduce the levels of inter-locus sexual conflict in the FEJ populations while proportionately improving investment towards reproduction (via increased relative testis size and AG). Breeding ecology differences might

appear only under conditions of resource limitation (smaller body size). For instance, the monogamous nature of the FEJs might be an outcome of both a lethargic pattern of movement (as a consequence of rapid development selection), leading to low energy resources at eclosion coupled with the short time available for mating.

To conclude, selection for rapid development alone likely reduces levels of inter-locus sexual conflict only in a body size dependent manner, while selection for early reproduction, in the absence of selection for rapid development, does not seem to lead to the evolution of lower inter-locus sexual conflict, in this system.

Tables

Table 5.1 Summary of ANOVA results on absolute testis size with selection and mating status as fixed factors, and block as a random factor. This design does not permit testing of random factors and interactions, and therefore, those results have been left out for brevity.

Effect	**df**	**MS**	***F***	***P***
Selection	3	0.0427	44.511	<0.001
Mating Status (st.)	1	0.0002	0.0981	0.774
Selection × Mating st.	3	0.0003	2.123	0.167

Table 5.2 Summary of ANOVA results on testis size (scaled by thorax length squared) with selection and mating status as fixed factors, and block as a random factor. This design does not permit testing of random factors and interactions, and therefore, those results have been left out for brevity.

Effect	**df**	**MS**	***F***	***P***
Selection	3	0.0006	19.099	<0.001
Mating Status (st.)	1	$5.12 \times 10^{\wedge -5}$	0.378	0.058
Selection × Mating st.	3	$2.63 \times 10^{\wedge -5}$	1.790	0.218

Table 5.3 Summary of ANOVA results on absolute AG with selection and mating status as fixed factors, and block as a random factor. This design does not permit testing of random factors and interactions, and therefore, those results have been left out for brevity.

Effect	**df**	**MS**	***F***	***P***
Selection	3	0.0205	111.397	<0.001
Mating Status (st.)	1	0.0439	231.226	<0.001
Selection × Mating st.	3	0.0006	2.054	0.176

Table 5.4 Summary of ANOVA results on AG size (scaled by thorax length squared) with selection and mating status as fixed factors, and block as a random factor. This design does not permit testing of random factors and interactions, and therefore, those results have been left out for brevity.

Effect	**df**	**MS**	***F***	***P***
Selection	3	0.0004	7.059	0.009
Mating Status (st.)	1	0.003	172.826	<0.001
Selection × Mating st.	3	$2.7 \times 10^{\wedge -5}$	0.942	0.459

Table 5.5 Summary of ANOVA results on reduction in accessory gland size and reduction in accessory gland size (scaled by thorax length squared) with selection as a fixed factor and block as a random factor. This design does not permit testing of random factors and interactions, and therefore, those results have been left out for brevity.

Effect	**df**	**MS**	***F***	***P***
Redn. (Absolute Size)	3	0.0012	2.054	0.176
Redn. (Scaled Size)	3	$7.43 \times 10^{\wedge -5}$	1.327	0.325

Table 5.6 Summary of ANOVA results for maturation time with selection regime and environment (yeasted or un-yeasted) as fixed factors. In this design, random factors and interactions with the same cannot be testes and have been left out for brevity.

Effect	**df**	**MS**	***F***	***P***
Selection	3	202.3021	31.5385	<0.001
Environment	1	0.0293	0.0075	0.9361
Selection × Environment	3	1.0483	0.3156	0.8139

Table 5.7 Summary of ANOVA results for copulation duration with selection regime and environment (yeasted or un-yeasted) as fixed factors. In this design, random factors and interactions with the same cannot be tested for significance and have been left out for brevity.

Effect	**df**	**MS**	***F***	***P***
Selection	3	38.2958	3.8676	0.0498
Environment	1	0.6157	0.1330	0.7394
Selection × Environment	3	5.2340	1.8987	0.2003

Figures

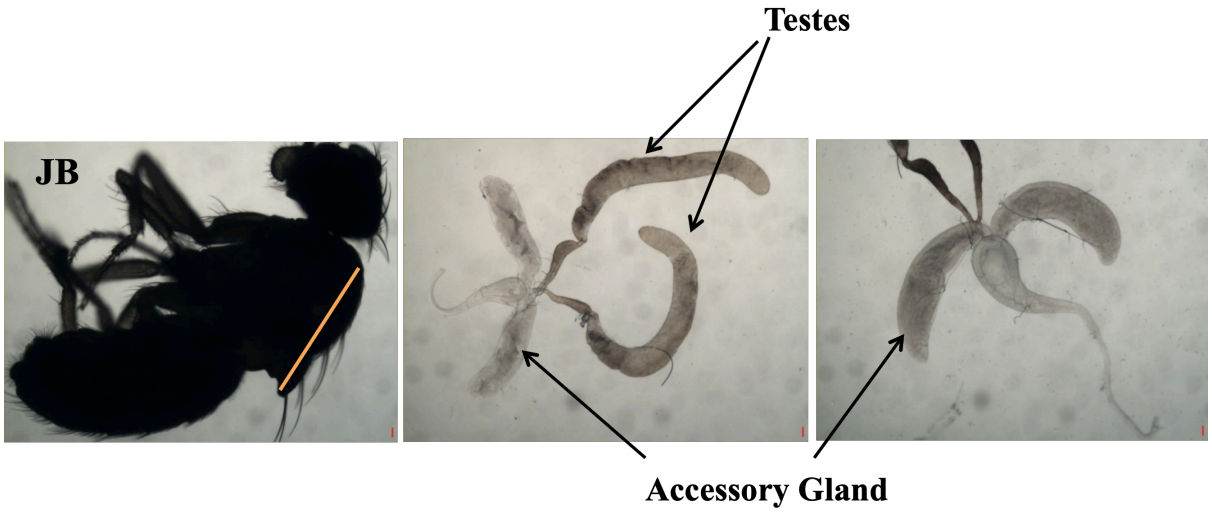


Fig 5.1 Image on the left end shows a JB male, with the orange bar representing the thorax length. The middle and right images show the relative position of the testes and the accessory glands.

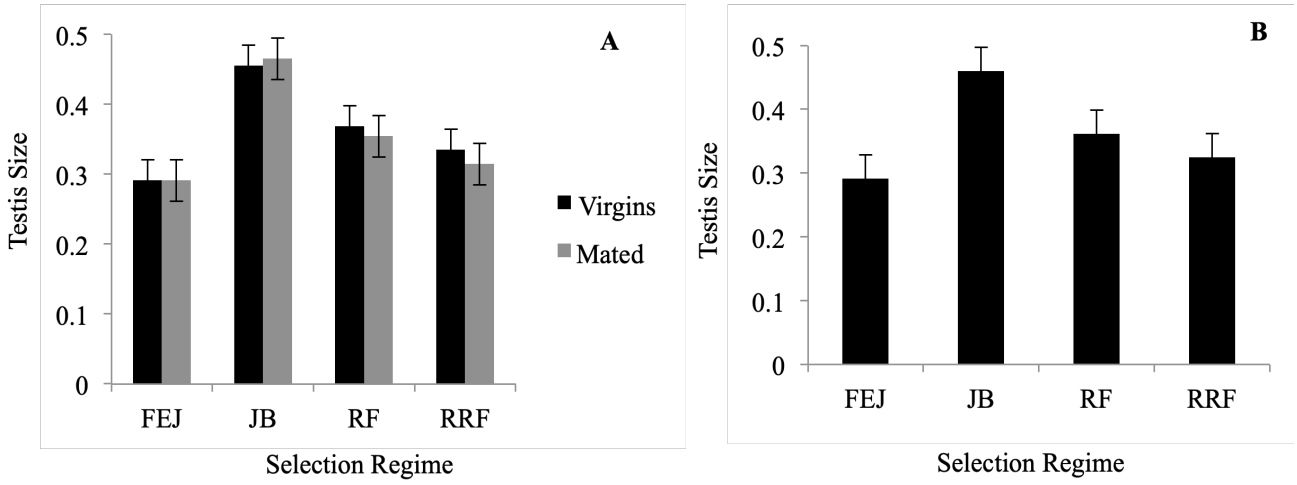


Fig 5.2 Mean absolute testis size of males from the four selection regimes as virgins and after mating once, averaged across four replicate populations (A), and testis size of males averaged across mating status and four replicate populations (B). Error bars are 95% confidence intervals around the means.

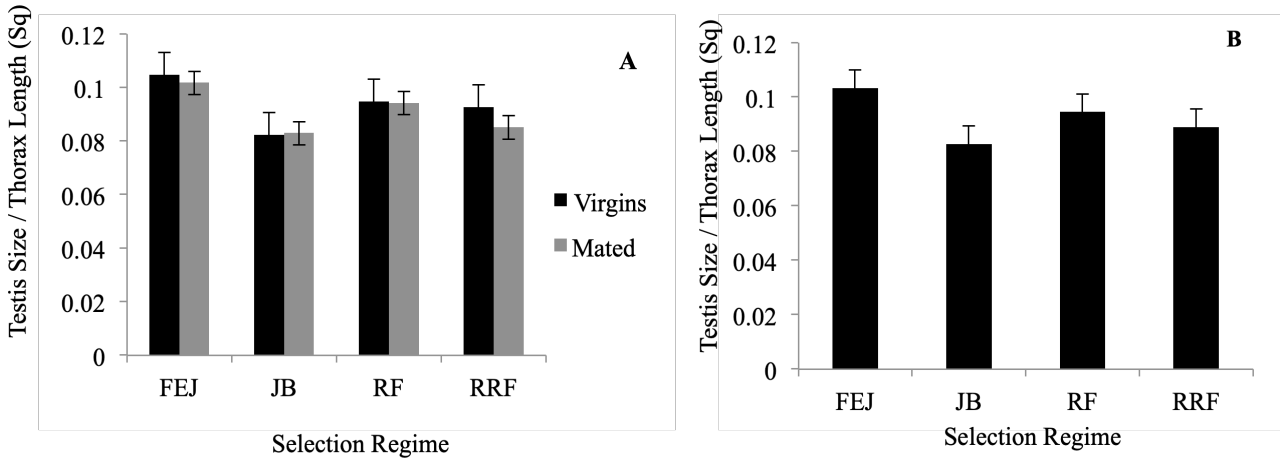


Fig 5.3 Mean testis size, scaled by thorax length squared, of males from the four selection regimes as virgins and after mating once, averaged across four replicate populations (A), and scaled testis size of males averaged across mating status and four replicate populations (B). Error bars are 95% confidence intervals around the means.

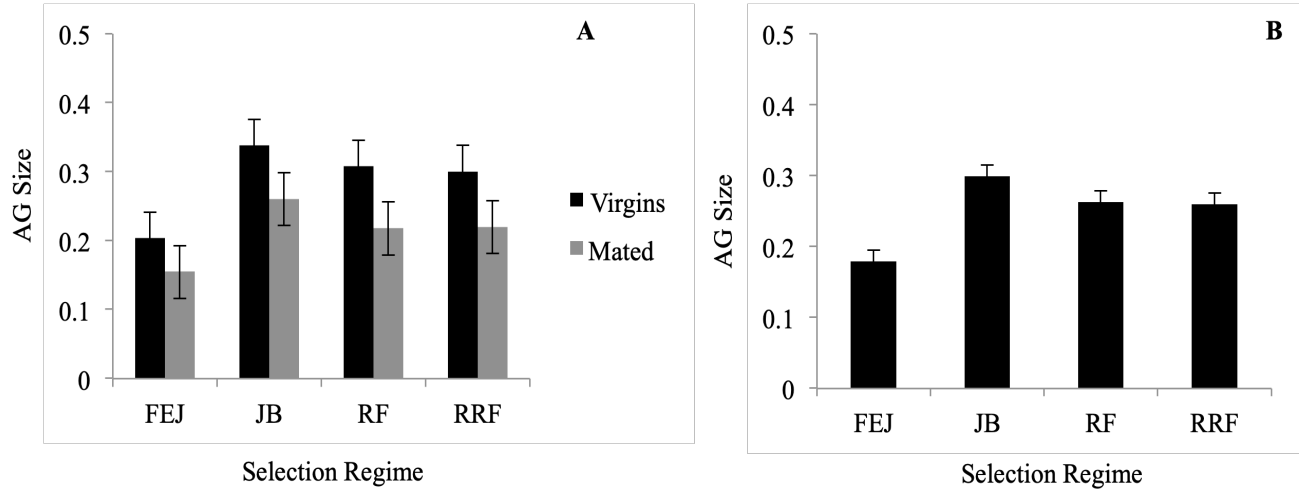


Fig 5.4 Mean absolute accessory gland size, of males from four selection regimes as virgins and after mating once, averaged across four replicate populations (A), and accessory gland of males averaged across mating status and four replicate populations (B). Error bars are 95% confidence intervals around the means.

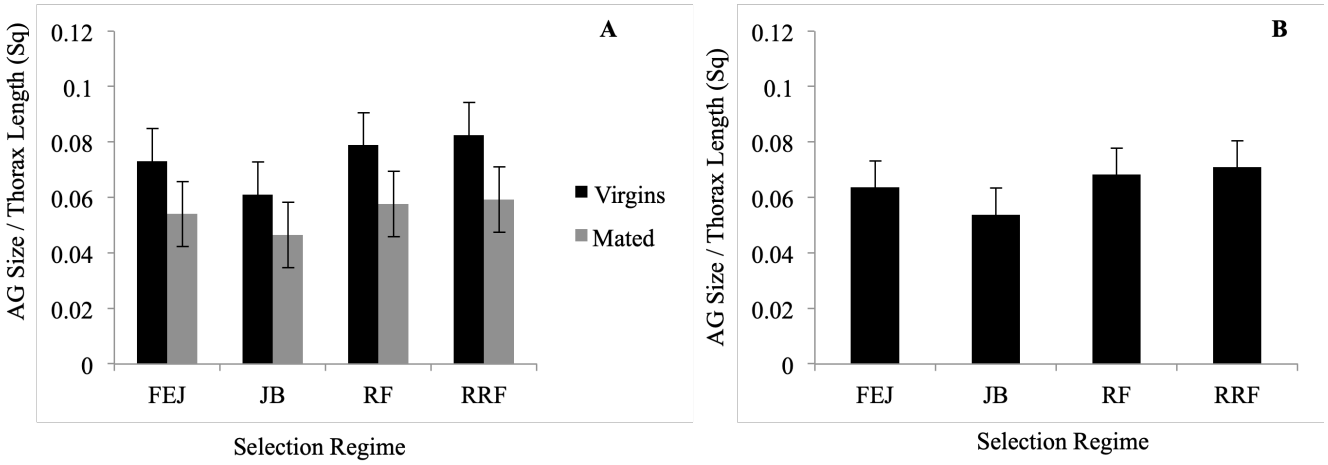


Fig 5.5 Mean accessory gland size, scaled by thorax length squared, of males from four selection regimes as virgins and after mating once, averaged across four replicate populations (A), and scaled accessory gland of males averaged across mating status and four replicate populations (B). Error bars are 95% confidence intervals around the means.

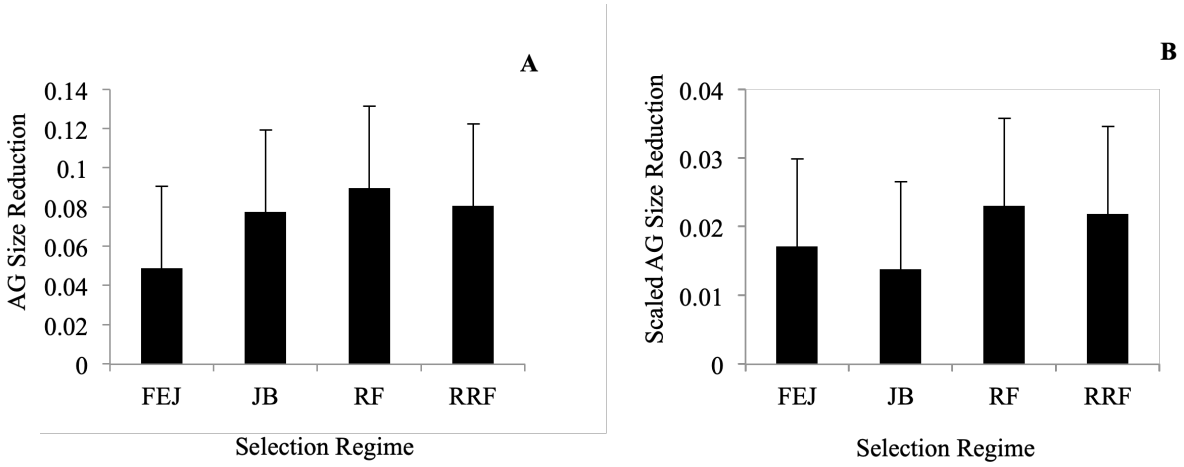


Fig 5.6 Mean reduction in accessory gland size (A), and accessory gland size reduction scaled by thorax length squared (B), of males from each selection regimes after mating, averaged across four replicate populations. Error bars are 95% confidence intervals around the means.

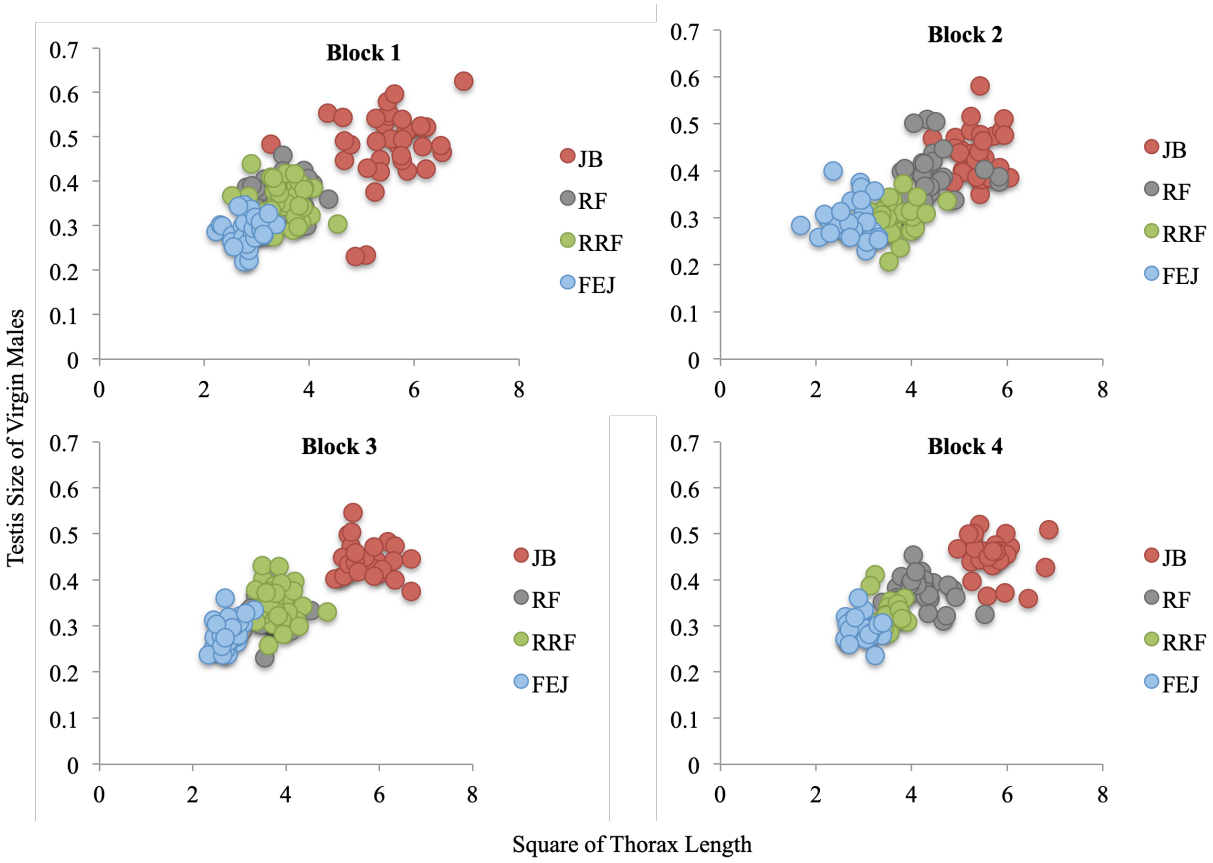


Fig 5.7 Scatter plot showing testis size of virgin males of the different selection regimes (y axis) and their corresponding squared thorax length (x axis) of the four replicate populations (blocks 1-4) of each selection regime.

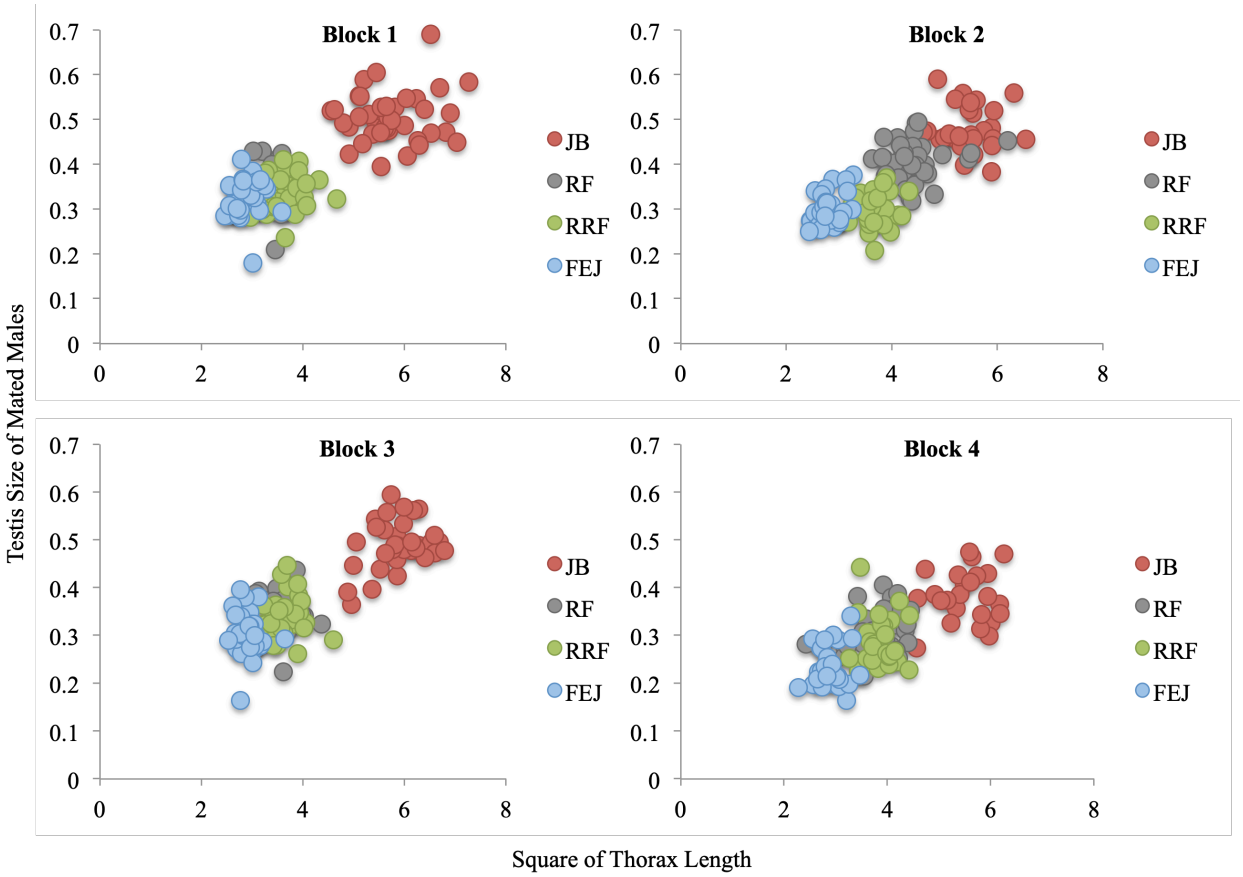


Fig 5.8 Scatter plot showing testis size of once-mated males of the different selection regimes (y axis) and their corresponding squared thorax length (x axis) of the four replicate populations (blocks 1-4) of each selection regime.

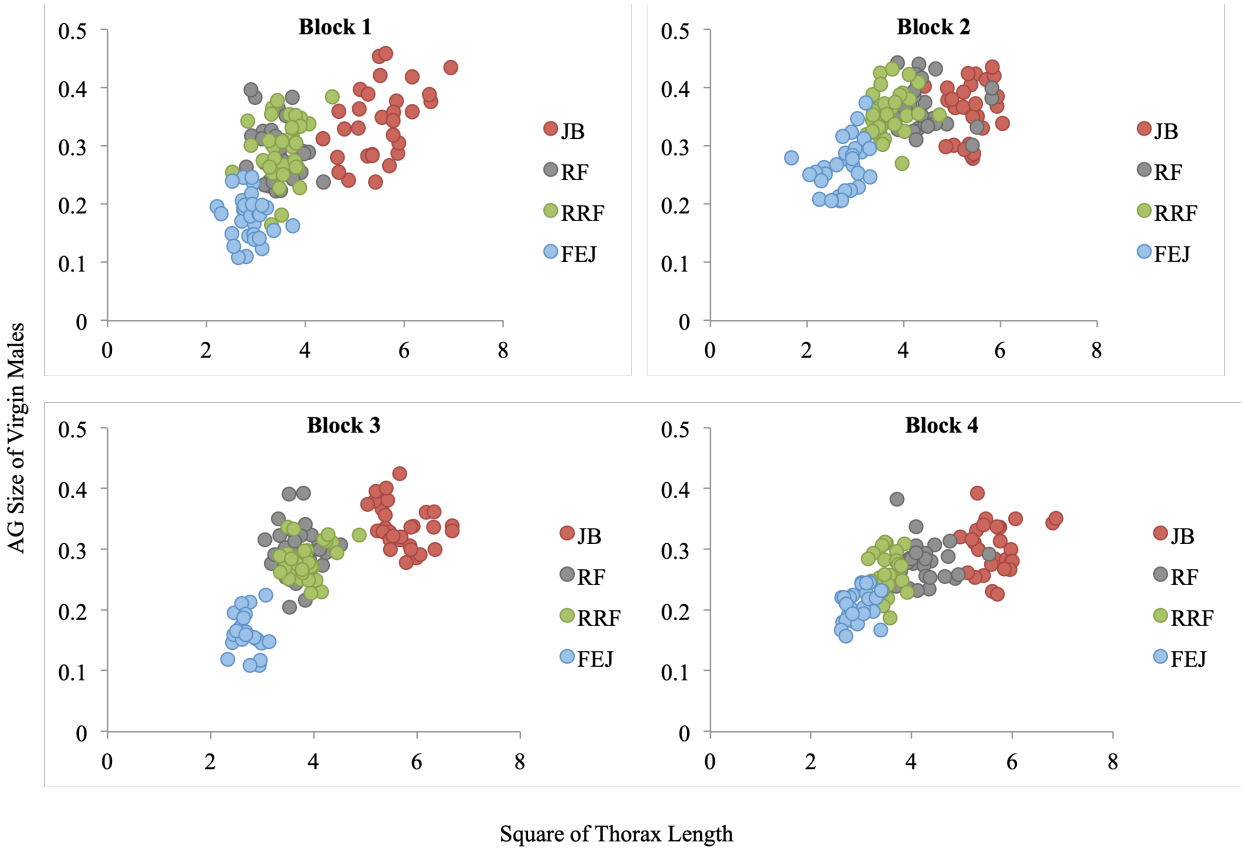


Fig 5.9 Scatter plot showing accessory gland size of virgin males of the different selection regimes (y axis) and their corresponding squared thorax length (x axis) of the four replicate populations (blocks 1-4) of each selection regime.

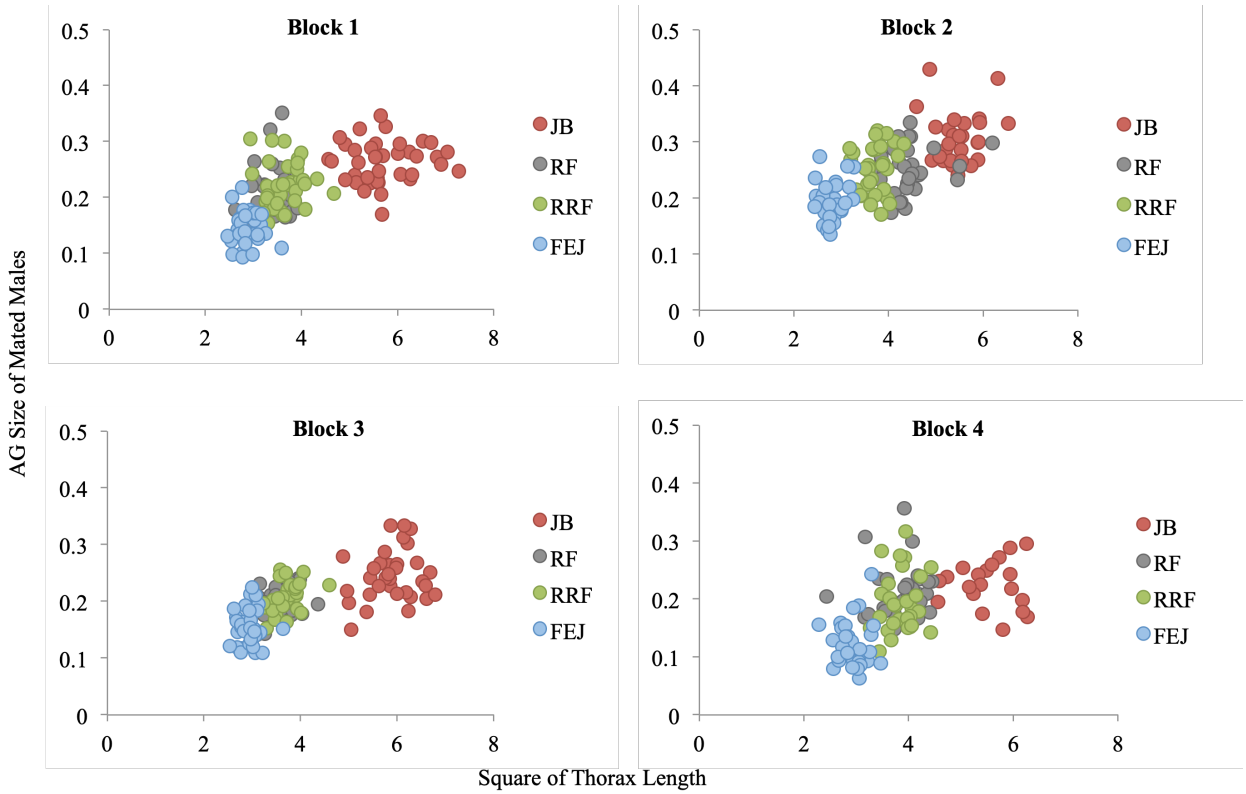


Fig 5.10 Scatter plot showing accessory gland size of once-mated males of the different selection regimes (y axis) and their corresponding squared thorax length (x axis) of the four replicate populations (blocks 1-4) of each selection regime.

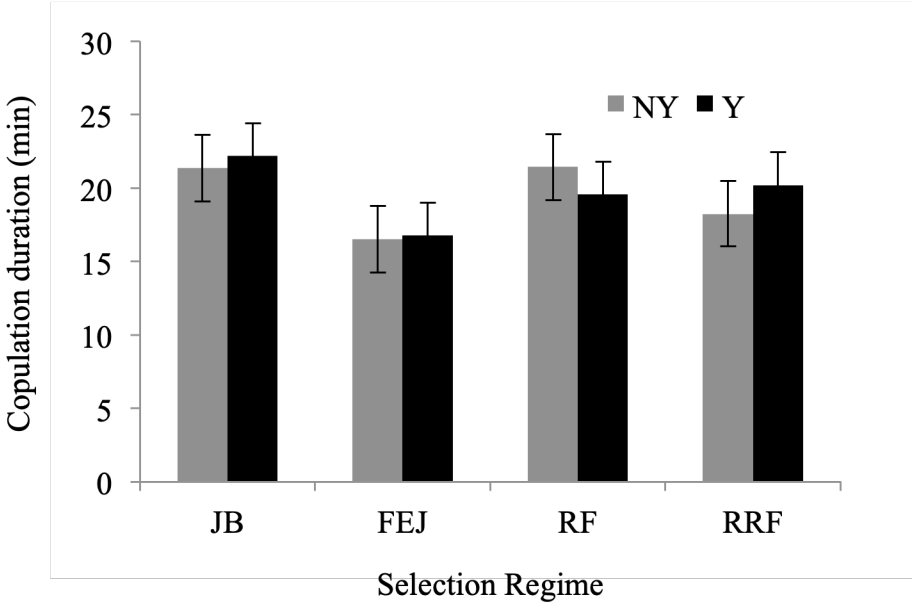


Fig. 5.11 Mean copulation duration (minutes) under yeasted and un-yeasted conditions in the JB, FEJ, RF and RRF populations, averaged across four replicate populations. Error bars are 95% confidence intervals around the means.

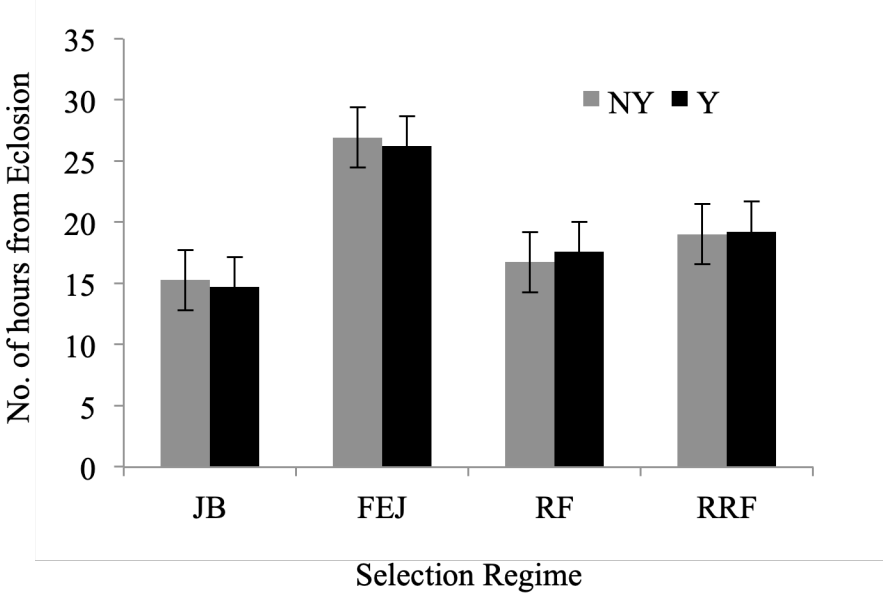


Fig. 5.12 Mean maturation time (hours) under yeasted and un-yeasted conditions in the JB, FEJ, RF and RRF populations, averaged across four replicate populations. Error bars are 95% confidence intervals around the means.

Chapter 6:

Discussion and Future Directions

Discussion

Inter-Locus Sexual Conflict and Life-Histories

The results from the work reported in this thesis suggest that despite great potential for age of reproduction affecting levels of sexual selection and inter-locus sexual conflict, the primary selective force leading to changes in sexually antagonistic traits in the set of populations of *D. melanogaster* studied by me is the strong directional selection on development time. At the outset, I had proposed three possible explanations for reduced levels of inter-locus sexual conflict in the FEJ populations.

1. *Body size based trade-off*: This was expected from the preliminary work conducted earlier (Mital 2013) and was confirmed in the work presented here. The reduction in body size as a result of selection for rapid development adequately explains the majority of my results pertaining to reduced levels of inter-locus sexual conflict in the FEJ populations, relative to the ancestral JB controls.
2. *Shorter breeding time might lead to lower sexual selection, less need for manipulation by males and, therefore, lower inter-locus sexual conflict*. This was not supported by my results as no differences were seen between RFs and RRFs in the traits examined.
3. *Change in inter-locus sexual conflict, i.e. a change in the nature of which traits contribute to fitness loss in females*. Females from the FEJ and RRF populations might not experience fitness losses upon manipulation by males due to their short effective adult life and might forego defenses against such manipulations. There was no clear support for this speculation either, although there is some suggestive evidence supporting this hypothesis.

These hypotheses, along with an examination of my results in the ambit of life-history selection, are discussed in detail below.

The FEJ and JB populations provide a useful system with which to examine the interaction between selection for life-history related traits and sexual selection, and this system is unique in its feature of exhibiting changes in inter-locus sexual conflict related traits, with those changes being driven primarily by the strong, primary selection for rapid development, despite the large potential for lowered sexual selection in the FEJ, compared to the ancestral controls, the JBs.

I found many features of male manipulation of female behaviour, as well as female resistance to such manipulations, to be primarily a function of the selection for rapid development (Chapter 4), perhaps explained by body size differences, while some features were not (Chapter 5). Specifically, increase in mortality of females post mating, their resistance to post-mating harm, and female fecundity boost post mating, corresponded well with body size differences, whereas differences in age at reproduction did not seem to affect these traits at all. Testis size was disproportionately smaller in the JB than the FEJs, while no differences were seen between RFs and RRFs in these traits. A synergistic effect of selection for rapid development and early reproduction in bringing down inter-locus sexual conflict in these populations is, thus, indicated. Most probably, the small and lethargic adult FEJs result in the breeding system becoming effectively monogamous during their three-day adult period, which may not be not true of the RRFs. Selection for rapid development may have reduced sexual selection via the evolution of adult traits, and the short breeding time could have provided the conditions for differences in sexual selection to operate.

Other studies have similarly looked at the effects of interaction of sexual selection and selection for life-history related traits, such as Hollis et al (2017), wherein selection for short generation times was superimposed on selection for either random monogamy or polygamy. They found high levels of sexual selection (polygamous populations) to indirectly select for rapid development and faster maturation compared to populations experiencing low sexual selection (monogamous populations) (Hollis et al, 2017), complementing the work presented here. Similarly, selection for late life reproduction has also provided evidence for tradeoffs in male competitive ability between early and late life (Service, 1993, Service and Fales, 1994, Service and Vossbrink, 1996), demonstrating that differences in the timing of effective reproduction in populations maintained under discrete generations can mediate the differential evolution of age specific patterns of expression of reproduction related traits, even those involved in male-female interactions, such that resource investment is optimized to maximize Darwinian fitness under existing constraints. Resource allocation and tradeoffs between fitness related traits are central to the study of life-histories and how they are shaped by selection (van Noordwijk and de Jong, 1986), and examples of this interaction between selection for life-history related traits and sex-specific selection exist in the experimental evolution literature, as discussed below. Kwan et al (2008) conjectured that since males from desiccation resistant *D. melanogaster* populations were inherently less tolerant to desiccation stress than females from those populations, male reproductive success would be achieved posthumously, and selection for early maturation and mating in males could be in conflict with selection for survival and late life reproduction in females. In those populations, strong selection for fertilization success of males (for instance, through high sperm competitive ability) could even be trading off with selection for male life-span. In fact, Darwin (1871) suggested that sexual selection may be a slow process

due to weak selection on traits in certain seasons, such as exaggerated male traits. Wallace (1871) also advocated for there to be selection against exaggerated male traits in certain life stages. The manner in which life-histories can constrain or reinforce the optimization of traits under survival and reproduction is the primary framework within which adaptation to different ecologies needs to be studied (Bonduriansky et al 2008), and the FEJ-JB system has allowed a nuanced examination of such adaptation.

It is, therefore, worth interpreting our results in the context of the FEJ life-history, which consists of a very short breeding duration compared to the JB. Based on the results from Chapters 3, 4 and 5, I conclude that the reduction in female resistance to male induced post mating harm has been a result of either co-evolution with smaller, less manipulative males, or is simply an austerity measure by females that have limited resources available to invest in traits that do not result in a significant improvement in fitness, as body size appears to correspond well with the female fitness trait values.

However, given that the two primary post mating responses in females are an immediate increase in egg output and higher mortality (Wolfner 1997, 2009), FEJ females might even be selected to forego resistance to mate manipulation as, in the context of their need to survive for only three days post mating and to lay a relatively large number of eggs on the third day, these male-induced changes would improve their fitness rather than depress it. These manipulative traits, therefore, may no longer be under conflict in the FEJ populations, unlike in the JBs where females need to survive for almost 11 days before eggs to initiate the next generation are collected. Results from Chapter 5 (Fig 5.3) reveal disproportionately smaller JB testis than those of the other selection regimes, with FEJs having the largest testis size relative to body size. The significant difference between (scaled) testis sizes between JB and FEJ indicates a greater

relative investment towards reproduction by the FEJ males as compared to the JB males. Moreover, FEJ females undergo substantially higher mortality post mating, compared to the JB females when both types of females mate with males from their selection regimes (Mital 2013). Given the FEJ life-history, as explained above, such an evolutionary change in the relative investment made towards reproduction would make sense. Perhaps fitness benefits of manipulation are accrued by females in FEJ populations, as argued above, and proportionally larger AG sizes in FEJ males (compared to JBs) would then be expected. I did find a trend towards larger AG sizes in the FEJ compared to JBs, and further research is necessary for this hypothesis to be adequately tested. Perhaps, a minimum absolute investment in these organs is to achieve any reproductive success, thus necessitating increased proportional investment in case of the FEJs. The absolute AG sizes of RFs and RRFs were very similar to that of JBs although they are smaller. Perhaps, the investment pattern in RFs and RRFs has not changed, being ancestrally closer to the FEJs, and the release from the constraints of rapid development allowed for such large AGs to evolve. In the life-history context, similarly, it would be in the interest of the JB males to be relatively less manipulative towards their partners, while still selecting JB females to maintain high resistance to mate manipulative harm in order to survive till day 21 from egg collection, explaining the relatively lower investment towards reproduction.

Additionally, the absolute differences in the FEJ and JB testis and AG sizes explain the earlier results obtained from studies with this system that show FEJs as having lower levels of inter-locus sexual conflict than JBs. Furthermore, earlier work from this lab has also reported lower Acp transcription levels in the FEJs compared to the JBs (Satish 2010, P Dey and A Joshi, *unpubl. data*). Therefore, despite relatively increased investment in reproduction, the absolute constraints imposed by strong selection for rapid development may have resulted in weaker FEJ

males, and consequently less resistant FEJ females, as compared to JBs. Interestingly, between RFs and RRFs, no such difference was seen despite differences in their life-histories, especially with regard to their effective adult lifespan.

The lifting of the selection for rapid development allowed me to check if the RRF females (which would experience the same fitness benefits of male manipulation as the FEJs) have lower resistance in comparison to the RF females, in terms of a fecundity boost and survivorship. Since I did not find any differences between them (Chapters 4, 5), even though this hypothesis is an interesting possibility, it warrants rejection in the absence of any further evidence. Further, no fitness loss or gain to females, in terms of fecundity up till four-five days of adult life, was seen in the FEJ or RRF females, with egg output being similar after mating with larger, more manipulative males as well as with smaller males (Figs. 4.5, 4.7).

A question then arises as to whether the co-evolutionary arms-race between males and females is itself conserved? If the nature of female fitness loss is changed, via life-history manipulations, why is inter-locus sexual conflict not affected? Perhaps, three days of adult life is still too long and investigating this phenomenon in populations such as the ACOs (Chippindale et al 1997a) which are large, outbred *D. melanogaster* populations selected for rapid development and very early reproduction (~24 hours as adults) might yield more insight. For instance, in the work demonstrating changes in male reproductive investment using populations selected for different ages at reproduction, the difference in the adult age at reproduction between the selection regimes was about nine weeks (Service 1993, Service and Fales 1994, Service and Vossbrink 1996), compared to only one week between the JB and FEJ populations.

It is also worthwhile to consider that females experiencing no fitness loss due to male mate manipulation may not be specific only to the FEJ type of life-history. Although typical theories on sex roles and research on inter-locus sexual conflict consider a male driven sexually antagonistic co-evolutionary process, females are not passive participants. Being a player in the 'arms race', females would also have some control over the evolutionary fate of these traits, i.e., they may not just be 'reacting' to 'active' adaptations by males (Green and Madjidian 2011). The very existence of polyandry in such a large number of insects indicates that direct benefits might be gained by multiple matings by females as well (Arnqvist and Nillsson 2000). It is perhaps more prudent to view the phenomenon of inter-locus sexual conflict as not only one that causes females to experience fitness loss, with them evolving counter-adaptations to these male manipulations, but rather that the context of the ecology and life-history of the population under study may drastically modify these interactions, their effects on the fitness of the two sexes, and the eventual escalation or amelioration of the 'conflict' in these interactions. It may well be worth-while to reconsider the 'conflict' status of traits that have traditionally been considered to trigger sexually antagonistic co-evolution.

Results from Chapter 2, in many ways, provide evidence for opportunities for selection for lower inter-locus sexual conflict, as well as evidence of lower conflict, which are difficult to disentangle. For instance, low courtship rate could be an outcome of less competition among males (Rice 1996) and, therefore, lower inter-locus sexual conflict, but could also be seen as causing reduction in inter-locus sexual conflict. Any developmental change causing males to become less manipulative is likely to give rise to the evolution of lower levels of female defense. Additional reduction in inter-locus sexual conflict would be expected in the FEJ, then, which would be reflected in their relative AG size. However, this was not found (Chapter 5), or such a

possibility reflected in the other traits investigated here (Chapter 4). Perhaps the FEJ flies have reached their lower limit on mate manipulation and resistance, with further reduction constrained by natural selection. In fact, the relatively larger testes and accessory glands of FEJs suggest that a disproportionately larger investment in these organs may be required to attain any reproductive fitness, given the large decrease in their body size.

Inbreeding

It is necessary to establish if inbreeding depression and random genetic drift might have brought about changes in trait values, especially while exploring fitness related traits that show reduction in trait value over the course of experimental evolution studies. For instance, Rice and Holland (2005) extensively needed to discuss and refute the role of smaller body size and inbreeding depression as explanations for reduction in male-induced harm and female resistance to that harm in their monogamy adapted populations. The study of reverse selection regimes an be very suggestive on the issue of inbreeding in laboratory adapted populations. Inbreeding can cause an accumulation of recessive deleterious mutations in homozygous form in populations, giving rise to overall lower fitness of individuals. The number of generations of selection in the FEJ has been so great that, even with a large effective population size, it would not be surprising to find accumulation of a number of such deleterious mutations. However, monogamy can partly ameliorate this constraint by making family sizes more similar, especially for males. Given the slow response found in the relaxed selection regimes I studied, it is worth considering the possibility of drift like effects in producing less harmful males, and less resistant females in FEJs. Work reported in Chapter 4 on male induced toxicity towards females, and female

resistance to that toxicity in RFs and RRFs, revealed that body size differences largely explained these effects. Inbreeding or drift like effects would have resulted in lower trait values for fitness related traits than what would be expected based on their adult body size. I believe that although many generations of strong directional selection may have resulted in fixation of alleles for rapid development, reducing the potential for a response in case of relaxation (as seen in Chapter 3), it has not been accompanied by a similar inbreeding or drift-induced loss in genetic variation for traits associated with sex specific fitness.

Future Directions

Perhaps one of the first of the subsequent studies on this system should be directed towards establishing the differences in levels of sexual selection between the RFs and the RRFs, if there are any. Although I have so far used courtship and mating rates (over their effective lifetimes) to establish the degree of sexual selection operating in the study populations, a more accurate estimate could be obtained by measuring the variances of male and female reproductive success. A higher optimum mating rate for males than for females leads to greater overall fitness benefit with each additional mating for males, as compared to females, resulting in higher variance in male reproductive success than in female reproductive success. Although mating rates suggest that FEJ males mate only once, on average, we do not know if the instances of mating being noted are from some FEJ males which are better at attaining mating, that mate more than once whereas others do not mate at all. Although the overall behaviour of these flies makes this an unlikely possibility, it is nevertheless worthwhile to establish if the reproductive fitness variances

are congruent for the two sexes in FEJs, and to what extent they differ in case of JBs, RFs and RRFs.

There are about 200Acps that have been recognized in various *D. melanogaster* alone, with a high degree of redundancy in their functions (Sirot et al 2014). This itself is indicative of an ongoing co-evolutionary arms race between the sexes, with males being selected to evolve newer proteins to perform the same functions that females have evolved resistance against. Given the resource constraint as well as the established reduction of inter-locus sexual conflict in the FEJs, the number and type of Acps is likely to have changed, relative to the ancestral JBs. Moreover, there are proteins that are directed towards improving sperm defense and offense abilities. If the probability of securing a second mating is very low in the FEJ, investing in traits related to sperm defense and offense is likely to prove wasteful. Alternatively, one can argue that it is the presence of a relatively high sperm defense ability (part of which involves reduced susceptibility of the female to re-mating) that lowers the probability of re-mating in the FEJ. Arguing along the same lines, there would be little to no need for any sperm offense ability in the FEJs. All these are traits worth investigating in the FEJ, RF, RRF and JB populations, given their different requirements based on age of reproduction and resource availability. Information on the complement of Acps produced, and specific defenses mounted against them by females of these populations, will further improve our understanding of this system.

Finally, based on the results presented in Chapter 5, testis size has evolved to become proportionately larger, relative to body size, in the FEJ populations. Testis size is implicated in both sperm size and number, and has been known to evolve based on specific sperm competition related needs in laboratory populations of *D. melanogaster* (Pitnick 2001, Pitnick and Miller 2000, Snook 2005). The degree of sperm competition is directly affected by sperm morphology

in *D. melanogaster* and related species, and sperm number and size will not only be a surrogate for estimating the degree of sperm competition in these populations but will also help establish how these males allocate the limited resources towards reproduction. In the FEJ, egg size has been shown to be greater (B M Prakash and A Joshi, *unpublished data*), and although their fecundity is lower than JB, their fecundity per unit weight is higher than that of JBs (Prasad 2004, Ghosh-Modak 2009). Thus, there is a clear indication of a trade-off and change in the egg production strategy that has evolved in the FEJ females. However, FEJ males have so far only revealed larger proportionate testis size as a significant evolutionary change in the pattern of investment in reproduction related traits. In fact, they appear to invest minimally towards courtship, leaving gamete production as the primary reproduction related requirement. Is the entire reproductive investment in FEJs made only towards gamete production? If so, how does the ratio of sperm size to number in the FEJs compare with that of the JBs?

All this underscores the chief point that this thesis attempts to make, i.e. FEJ flies, for their ecology, are highly specialized and 'fit' in the suite of traits they exhibit, yielding maximal possible fitness. This includes traits related to rejection or acceptance of males by females, male pursuit of females, investment in Acps, resistance to these Acps by females, survival, fecundity, adult immunity (Modak et al 2009), and courtship and mating rates. Sexual selection in the FEJ populations is not of the same nature as it is in the JB populations and sexually antagonistic traits, just like trait correlations, are labile and make sense only in the light of the life-history of the organism. This work has helped improve our understanding of the relationship between strong selection for rapid development and age at reproduction in terms of mediating lower levels of sexually antagonistic co-evolution between males and females. However, many questions remain unanswered and further exploration will, hopefully, answer some of them and

also open new avenues of research in the field of life-history evolution and male-female co-evolution.

List of References

Amitin, E. G., & Pitnick, S. (2007). Influence of developmental environment on male□and female□mediated sperm precedence in *Drosophila melanogaster*. *Journal of Evolutionary Biology*, **20**, 381-391.

Andersson, M. (1994). *Sexual Selection*. Princeton University Press, Princeton.

Archer, M. A., Phelan, J. P., Beckman, K. A., & Rose, M. R. (2003). Break down in correlations during laboratory evolution. II. Selection on stress resistance in *Drosophila* populations. *Evolution*, **57**, 536-543.

Arnqvist, G., & Nilsson, T. (2000). The evolution of polyandry: multiple mating and female fitness in insects. *Animal Behaviour*, **60**, 145-164.

Arnqvist, G., & Rowe, L. (2013). *Sexual Conflict*. Princeton University Press, Princeton.

Bakker, K. (1959). Feeding period, growth and pupation in larvae of *Drosophila melanogaster*. *Entomologia Experimentaliset Applicata*, **2**, 171-186.

Bangham, J., Chapman, T., & Partridge, L. (2002). Effects of body size, accessory gland and testis size on pre-and post-copulatory success in *Drosophila melanogaster*. *Animal Behaviour*, **64**, 915-921.

Bateman, A. J. (1948). Intra-sexual selection in *Drosophila*. *Heredity*, **2**, 349-368.

Bonduriansky, R., & Chenoweth, S.F. (2009). Intralocus sexual conflict. *Trends in Ecology and Evolution*, **24**, 280-288.

Bonduriansky, R., Maklakov, A., Zajitschek, F., & Brooks, R. (2008). Sexual selection, sexual conflict and the evolution of ageing and life span. *Functional Ecology*, **22**, 443-453.

Borash, D. J., Gibbs, A. G., Joshi, A., & Mueller, L. D. (1998). A genetic polymorphism maintained by natural selection in a temporally varying environment. *The American Naturalist*, **151**, 148-156.

Bretman, A., Fricke, C., & Chapman, T. (2009). Plastic responses of male *Drosophila melanogaster* to the level of sperm competition increase male reproductive fitness. *Proceedings of the Royal Society of London B: Biological Sciences*, **276**, 1705-1711.

Bull, J. J., &Charnov, E.L. (1985). On irreversible evolution. *Evolution*, **39**, 1149-1155.

Byrne, P. G., & Rice, W. R. (2006). Evidence for adaptive male mate choice in the fruit fly *Drosophila melanogaster*. *Proceedings of the Royal Society of London B: Biological Sciences*, **273**, 917-922.

Chapman, T., Liddle, L. F., Kalb, J. M., Wolfner, M. F., & Partridge, L. (1995).Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. *Nature*, **373**, 241-244.

Chapman, T. (2001). Seminal fluid-mediated fitness traits in *Drosophila*. *Heredity*, **87**, 511.

Chapman, T., Arnqvist, G., Bangham, J., & Rowe, L. (2003). Sexual conflict. *Trends in Ecology and Evolution*, **18**, 41-47.

Chechi, T. S., Syed, Z. A., & Prasad, N. G. (2017). Virility does not imply immensity: testis size, accessory gland size and ejaculate depletion pattern do not evolve in response to experimental manipulation of sex ratio in *Drosophila melanogaster*. *Journal of Insect Physiology*, **98**, 67-73.

Chen, P. S., Stumm-Zollinger, E., Aigaki, T., Balmer, J., Bienz, M., &Böhlen, P. (1988). A male accessory gland peptide that regulates reproductive behavior of female *D. melanogaster*. *Cell*, **54**, 291-298.

Chippindale, A. K., Hoang, D. T., Service, P. M., &Rose, M. R. (1994). The evolution of development in *Drosophila melanogaster* selected for postponed senescence. *Evolution.* **48***,* 1880-1899.

Chippindale, A. K., Chu, T. J., & Rose, M. R. (1996). Complex trade□offs and the evolution of starvation resistance in *Drosophila melanogaster*. *Evolution*, **50**, 753-766.

Chippindale, A. K., Alipaz, J. A., Chen, H. W., & Rose, M. R. (1997)-a. Experimental evolution of accelerated development in *Drosophila*. 1. Developmental speed and larval survival. *Evolution*, **51**, 1536-1551.

Chippindale, A. K., Leroi, A. M., Saing, H., Borash, D. J., & Rose, M. R. (1997)-b. Phenotypic plasticity and selection in *Drosophila* life history evolution. 2. Diet, mates and the cost of reproduction. *Journal of Evolutionary Biology*, **10**, 269-293.

Chippindale, A.K., Gibbs, A. G., Sheik, M., Yee, K. J., Djawdan, M., Bradley, T. J., & Rose, M. R. (1998). Resource acquisition and the evolution of stress resistance in *Drosophila melanogaster*. *Evolution*, **52**, 1342-1352.

Chippindale, A. K., Gibson, J. R., & Rice, W. R. (2001). Negative genetic correlation for adult fitness between sexes reveals ontogenetic conflict in *Drosophila*. *Proceedings of the National Academy of Sciences*, **98**, 1671-1675.

Cordts, R., & Partridge, L.(1996). Courtship reduces longevity of male *Drosophila melanogaster*. *Animal Behaviour*, **52**, 269-278.

Crudgington, H. S., Beckerman, A. P., Brüstle, L., Green, K., & Snook, R. R. (2005). Experimental removal and elevation of sexual selection: does sexual selection generate manipulative males and resistant females? *The American Naturalist*, **165**, 72-87.

Darwin, C. R. (1871). *The Descent of Man and Selection in Relation to Sex*. John Murray, London.

Djawdan, M., Sugiyama, T. T., Schlaeger, L. K., Bradley, T. J., & Rose, M. R. (1996). Metabolic aspects of the trade-off between fecundity and longevity in *Drosophila melanogaster*. *Physiological Zoology,* **69**, 45-164.

Dow, M. A., & von Schilcher, F. (1975). Aggression and mating success in *Drosophila melanogaster*. *Nature*, **254**, 511.

Edward, D. A., & Chapman, T. (2012). Measuring the fitness benefits of male mate choice in *Drosophila melanogaster*. *Evolution*, **66**, 2646-2653.

Edward, D. A., & Chapman, T. (2012). Sex□specific effects of developmental environment on reproductive trait expression in *Drosophila melanogaster*. *Ecology and Evolution*, **2**, 1362-1370.

Edward, D. A., Fricke, C., & Chapman, T. (2010). Adaptations to sexual selection and sexual conflict: insights from experimental evolution and artificial selection. *Philosophical transactions of the Royal Society of London. Series B, Biological Sciences*, **365**, 2541-8.

Emlen, D. J. & Oring, L. W. (1977). Ecology, sexual selection, and the evolution of mating systems. *Science*, **197**, 215-223.

Falconer, D. S., & Mackay, T. F. C. (1996). Hardy-Weinberg Equilibrium. *Introduction to Quantitative Genetics, 4th edition.* Addison Wesley Longman, Harlow, Essex, UK, 5-18.

Fedorka, K. M., Linder, J. E., Winterhalter, W., & Promislow, D. (2007).Post-mating disparity between potential and realized immune response in *Drosophila melanogaster*. *Proceedings of the Royal Society of London B: Biological Sciences*, **274**, 1211-1217.

Fisher, R. A. (1915). The evolution of sexual preference. *The Eugenics Review*, **7**, 184-191.

Fowler, K., & Partridge, L. (1989). A cost of mating in female fruitflies. *Nature*, **338**, 760.

Friberg, U., & Arnqvist, G. (2003). Fitness effects of female mate choice: preferred males are detrimental for *Drosophila melanogaster* females. *Journal of Evolutionary Biology*, **16**, 797-811.

Fricke, C., Bretman, A., & Chapman, T. (2010). Female nutritional status determines the magnitude and sign of responses to a male ejaculate signal in *Drosophila melanogaster*. *Journal of Evolutionary Biology*, **23**, 157-165.

Gavrilets, S., Arnqvist, G., & Friberg, U. (2001). The evolution of female mate choice by sexual conflict. *Proceedings of the Royal Society of London B: Biological Sciences*, **268**, 531-539.

Ghosh-Modak, S. G. (2009). *Evolution of life-history traits, canalization and reproductive isolation in laboratory populations of Drosophila melanogaster selected for faster pre-adult development and early reproduction*. Ph D. Thesis, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, India.

Ghosh, S. M., & Joshi, A. (2012). Evolution of reproductive isolation as a by□product of divergent life□history evolution in laboratory populations of *Drosophila melanogaster*. *Ecology and Evolution*, **2**, 3214-3226.

Gilchrist, A. S., & Partridge, L. (2000). Why it is difficult to model sperm displacement in *Drosophila melanogaster*: the relation between sperm transfer and copulation duration. *Evolution*, **54**, 534-542.

Green, K. K., & Madjidian, J. A. (2011). Active males, reactive females: stereotypic sex roles in sexual conflict research? *Animal Behaviour*, **81**, 901-907.

Harshman, L. G., & Prout, T. (1994). Sperm displacement without sperm transfer in *Drosophila melanogaster*. *Evolution*, **48**, 758-766.

Harshman, L. G., & Hoffmann, A. A. (2000). Laboratory selection experiments using *Drosophila*: what do they really tell us? *Trends in Ecology and Evolution*, **15**, 32-36.

Heifetz, Y., Lung, O., Frongillo Jr, E. A., & Wolfner, M. F. (2000). The *Drosophila* seminal fluid protein Acp26Aa stimulates release of oocytes by the ovary. *Current Biology*, **10**, 99-102.

Herndon, L. A., &Wolfner, M. F. (1995). A *Drosophila* seminal fluid protein, Acp26Aa, stimulates egg laying in females for 1 day after mating. *Proceedings of the National Academy of Sciences*, **92**, 10114-10118.

Holland, B., & Rice, W. R. (1998). Perspective: chase□away sexual selection: antagonistic seduction versus resistance. *Evolution*, **52**, 1-7.

Holland, B., & Rice, W. R. (1999). Experimental removal of sexual selection reverses intersexual antagonistic co-evolution and removes a reproductive load. *Proceedings of the National Academy of Sciences*, **96**, 5083-5088.

Hollis, B., Houle, D., Yan, Z., Kawecki, T. J., &Keller, L. (2014). Evolution under monogamy feminizes gene expression in *Drosophila melanogaster*. *Nature Communications*, **5**, 3482.

Hollis, B., Keller, L., & Kawecki, T. J. (2017). Sexual selection shapes development and maturation rates in *Drosophila*. *Evolution*, **71**, 304-314.

Houle, D., & Rowe, L. (2002). Natural selection in a bottle. *The American Naturalist*, **161**, 50-67.

Imroze, K., & Prasad, N. G. (2011). Mating with large males decreases the immune defence of females in *Drosophila melanogaster*. *Journal of Genetics*, **90**, 427-434.

Jones, A. G., Rosenqvist, G., Berglund, A., Arnold, S. J., & Avise, J. C. (2000).The Bateman gradient and the cause of sexual selection in a sex-role-reversed pipefish. *Proceedings of the Royal Society of London B: Biological Sciences*, **267**, 677-680.

Joshi, A., & Mueller, L. D. (1996). Density-dependent natural selection in *Drosophila*: trade-offs between larval food acquisition and utilization. *Evolutionary Ecology*, **10**, 463-474.

Joshi, A., Castillo, R. B., & Mueller, L. D. (2003). The contribution of ancestry, chance, and past and ongoing selection to adaptive evolution. *Journal of Genetics*, **82**, 147-162.

Kuijper, B., Stewart, A. D., & Rice, W. R. (2006). The cost of mating rises nonlinearly with copulation frequency in a laboratory population of *Drosophila melanogaster*. *Journal of Evolutionary Biology*, **19**, 1795-1802.

Kwan, L., Bedhomme, S., Prasad, N. G., & Chippindale, A. K. (2008). Sexual conflict and environmental change: trade-offs within and between the sexes during the evolution of desiccation resistance. *Journal of Genetics*, **87**, 383-394.

Linklater, J. R., Wertheim, B., Wigby, S., & Chapman, T. (2007). Ejaculate depletion patterns evolve in response to experimental manipulation of sex ratio in *Drosophila melanogaster*. *Evolution*, **61**, 2027-2034.

Liu, H., & Kubli, E. (2003). Sex-peptide is the molecular basis of the sperm effect in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, **100**, 9929-9933.

Long, T. A. F., Pischedda, A., Stewart, A. D., &Rice, W. R. (2009).A cost of sexual attractiveness to high-fitness females. *PLoS Biology*, **7**, e1000254.

Long, T. A. F., Pischedda, A., Nichols, R. V., & Rice, W. R. (2010). The timing of mating influences reproductive success in *Drosophila melanogaster*: implications for sexual conflict. *Journal of Evolutionary Biology*, ***23***, 1024-1032.

Luckinbill, L. S., & Clare, M. J. (1985). Selection for life span in *Drosophila melanogaster*. *Heredity*, **55**, 9.

Lüpold, S., Manier, M. K., Berben, K. S., Smith, K. J., Daley, B. D., Buckley, S. H., & Pitnick, S. (2012). How multivariate ejaculate traits determine competitive fertilization success in *Drosophila melanogaster*. *Current Biology*, **22**, 1667-1672.

Lüpold, S., Manier, M. K., Puniamoorthy, N., Schoff, C., Starmer, W. T., Luepold, S. H. B.,& Pitnick, S. (2016). How sexual selection can drive the evolution of costly sperm ornamentation. *Nature*, **7604**, 533-535.

Lüpold, S., & Pitnick, S. (2018). Sperm form and function: what do we know about the role of sexual selection? *Reproduction*, **5**, 229-243.

Maan, M. E., & Seehausen, O. (2011). Ecology, sexual selection and speciation. *Ecology Letters*, **14**, 591-602.

Manier, M. K., Belote, J. M., Berben, K. S., Novikov, D., Stuart, W. T., & Pitnick, S. (2010). Resolving mechanisms of competitive fertilization success in *Drosophila melanogaster*. *Science*, **328**, 354-357.

Manier, M. K., Belote, J. M., Berben, K. S., Lüpold, S., Ala-Honkola, O., Collins, W. F., & Pitnick, S. (2013). Rapid diversification of sperm precedence traits and processes among three sibling *Drosophila* species. *Evolution*, **67**, 2348-2362.

Mason, J. S., Rostant, W. G., & Chapman, T. (2016). Resource limitation and responses to rivals in males of the fruit fly *Drosophila melanogaster*. *Journal of Evolutionary Biology*, **29**, 2010-2021.

Mital, A. (2013). *Preliminary investigations into reproductive fitness and sexual conflict in Drosophila populations selected for rapid pre-adult development and early reproduction*. M.S. Thesis, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, India.

Modak, S. G., Satish, K. M., Mohan, J., Dey, S., Raghavendra, N., Shakarad, M., & Joshi, A. (2009). A possible tradeoff between developmental rate and pathogen resistance in *Drosophila melanogaster*. *Journal of Genetics*, **88**, 253-256.

Mueller, L. D. (1985). The evolutionary ecology of *Drosophila*. *Evolutionary Biology*, **19**, 37-98

Mueller, L. D., Joshi, A., Santos, M., & Rose, M. R. (2013). Effective population size and evolutionary dynamics in outbred laboratory populations of *Drosophila*. *Journal of Genetics*, **92**, 349-361.

Nandy, B., & Prasad, N. G. (2011). Reproductive behavior and fitness components in male *Drosophila melanogaster* are non-linearly affected by the number of male co-inhabitants early in adult life. *Journal of Insect Science*, **11**, 67.

Nandy, B., Joshi, A., Ali, Z. S., Sen, S., & Prasad, N. G. (2012). Degree of adaptive male mate choice is positively correlated with female quality variance. *Scientific Reports*, **2**, 447.

Nandy, B., Chakraborty, P., Gupta, V., Ali, S. Z., &Prasad, N. G. (2013)-a. Sperm competitive ability evolves in response to experimental alteration of operational sex ratio. *Evolution*, **67**, 2133-2141.

Nandy, B., Gupta, V., Sen, S., Udaykumar, N., Samant, M. A., Ali, S. Z., & Prasad, N. G. (2013)-b. Evolution of mate-harm, longevity and behaviour in male fruit flies subjected to different levels of inter-locus conflict. *BMC Evolutionary Biology*, **13**, 212.

Nandy, B., Gupta, V., Udaykumar, N., Samant, M. A., Sen, S., & Prasad, N. G. (2014). Experimental evolution of female traits under different levels of intersexual conflict in *Drosophila melanogaster*. *Evolution*, **68**, 412-425.

Nunney, L. (1996). The response to selection for fast larval development in *Drosophila melanogaster* and its effect on adult weight: an example of a fitness trade□off. *Evolution*, **50**, 1193-1204.

Parker, G. A. (1979). Sexual selection and sexual conflict. *Sexual Selection and Reproductive Competition in Insects*, Academic Press, Athens, Georgia.

Parker, G. A., & Partridge, L. (1998). Sexual conflict and speciation. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, **353**, 261-274.

Partridge, L., & Farquhar, M. (1983). Lifetime mating success of male fruit flies (*Drosophila melanogaster*) is related to their size. *Animal Behaviour*, **31**, 871-877.

Partridge, L., Fowler, K., Trevitt, S., & Sharp, W. (1986). An examination of the effects of males on the survival and egg-production rates of female *Drosophila melanogaster*. *Journal of Insect Physiology,* **32**, 925-929.

Partridge, L., Hoffmann, A., & Jones, J. S. (1987)-a. Male size and mating success in *D. melanogaster* and *D. pseudoobscura* under field conditions. *Animal Behaviour*, **35**, 468-476.

Partridge, L., Ewing, A., & Chandler, A. (1987)-b. Male size and mating success in *Drosophila melanogaster*: the roles of male and female behaviour. *Animal Behaviour*, **35**, 555-562.

Partridge, L., Green, A., & Fowler, K. (1987)-c. Effects of egg-production and of exposure to males on female survival in *Drosophila melanogaster*. *Journal of Insect Physiology*, **33**, 745-749.

Partridge, L., & Fowler, K. (1990). Non-mating costs of exposure to males in female *Drosophila melanogaster*. *Journal of Insect Physiology*, **36**, 419-425.

Phelan, J. P., Archer, M. A., Beckman, K. A., Chippindale, A. K., Nusbaum, T. J., & Rose, M. R. (2003). Breakdown in correlations during laboratory evolution. I. Comparative analyses of *Drosophila* populations. *Evolution*, **57**, 527-535.

Pitnick, S. (1991). Male size influences mate fecundity and remating interval in *Drosophila melanogaster*. *Animal Behaviour*, **41**, 735-745.

Pitnick, S. (1996). Investment in testes and the cost of making long sperm in *Drosophila*. *The American Naturalist*, **148**, 57-80.

Pitnick, S., & Miller, G. T. (2000). Correlated response in reproductive and life history traits to selection on testis length in *Drosophila hydei*. *Heredity*, **84**, 416.

Pitnick, S., Miller, G. T., Reagan, J., & Holland, B. (2001). Males' evolutionary responses to experimental removal of sexual selection. *Proceedings of the Royal Society of London B: Biological Sciences*, **268**, 1071-1080.

Pitnick, S., & García–González, F. (2002). Harm to females increases with male body size in *Drosophila melanogaster*. *Proceedings of the Royal Society of London B: Biological Sciences*, **269**, 1821-1828.

Prasad, N. G., Shakarad, M., Gohil, V. M., Sheeba, V., Rajamani, M., & Joshi, A. (2000). Evolution of reduced pre-adult viability and larval growth rate in laboratory populations of

Drosophila melanogaster selected for shorter development time. *Genetics Research*, **76**, 249-259.

Prasad, N. G., Shakarad, M., Anitha, D., Rajamani, M., & Joshi, A. (2001). Correlated responses to selection for faster development and early reproduction in *Drosophila*: the evolution of larval traits. *Evolution*, **55**, 1363-1372.

Prasad, N. G., Shakarad, M., Rajamani, M., & Joshi, A. (2003). Interaction between the effects of maternal and larval levels of nutrition on pre-adult survival in *Drosophila melanogaster*. *Evolutionary Ecology Research*, **5**, 903-911.

Prasad, N. G., & Joshi, A. (2003). What have two decades of laboratory life-history evolution studies on *Drosophila melanogaster* taught us? *Journal of Genetics*, **82**, 45-76.

Prasad, N. G. (2004). *Life-history evolution in laboratory populations of Drosophila melanogaster subjected to selection for faster development and early reproduction*. Ph D. Thesis, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, India.

Prasad, N. G., Bedhomme, S., Day, T., & Chippindale, A. K. (2006). An evolutionary cost of separate genders revealed by male-limited evolution. *The American Naturalist*, **169**, 29-37.

Rasband, W.S., (1997-2018ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/.

Rhen, T. (2000). Sex-limited mutations and the evolution of sexual dimorphism. *Evolution*, **54**, 37-43.

Rice, W. R. (1996). Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. *Nature*, **381**, 232-234.

Rice, W. R., & Holland, B. (1997). The enemies within: inter-genomic conflict, inter-locus contest evolution (ICE), and the intra-specific Red Queen. *Behavioral Ecology and Sociobiology*, **41**, 1-10.

Rice, W. R., & Holland, B. (2005). Experimentally enforced monogamy: inadvertent selection, inbreeding, or evidence for sexually antagonistic co-evolution? *Evolution*, **59**, 682-685.

Rice, W. R., Stewart, A. D., Morrow, E. H., Linder, J. E., Orteiza, N., & Byrne, P. G. (2006). Assessing sexual conflict in the *Drosophila melanogaster* laboratory model system. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, **361**, 287-299.

Ritchie, M. G. (2007). Sexual selection and speciation. *Annual Review of Ecology, Evolution and Systematics*, **38**, 79-102.

Roff, D. A. (1992).*The evolution of life histories*. Chapman and Hall, New York.

Rose, M. R. (1984). Laboratory evolution of postponed senescence in *Drosophila melanogaster*. *Evolution*, **38**, 1004-1010.

Rose, M. R., & Charlesworth, B. (1981). Genetics of life history in *Drosophila melanogaster*. II. Exploratory selection experiments. *Genetics*, **97**, 187-196.

Ruhmann, H., Wensing, K. U., Neuhalfen, N., Specker, J. H., & Fricke, C. (2016). Early reproductive success in *Drosophila* males is dependent on maturity of the accessory gland. *Behavioral Ecology*, **27**, 1859-1868.

Satish, K. M. (2010). *Reverse evolution and gene expression studies on populations of Drosophila melanogaster selected for rapid pre-adult development and early reproduction*. Ph D. Thesis, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, India.

Schaerer, L., Rowe, L., & Arnqvist, G. (2012). Anisogamy, chance and the evolution of sex roles. *Trends in Ecology and Evolution*, **27**, 260-264.

Schultzhaus, J. N., & Carney, G. E. (2017). Dietary protein content alters both male and female contributions to *Drosophila melanogaster* female post-mating response traits. *Journal of Insect Physiology*, **99**, 101-106.

Service, P. M. (1993). Laboratory evolution of longevity and reproductive fitness components in male fruit flies: mating ability. *Evolution*, **47**, 387-399.

Service, P. M., & Fales, A. J. (1994). Evolution of delayed reproductive senescence in male fruit flies: sperm competition. *Genetics and Evolution of Aging,* Rose, M. R., Finche, C. E., (eds), Springer, Dordrecht, 130-144.

Service, P. M. & Vossbrink, R. E. (1996). Genetic variation in "first" male effects on egg laying and re-mating by female *Drosophila melanogaster*. *Behavior Genetics*, **26**, 39-48.

Sgrò, C. M., Geddes, G., Fowler, K., & Partridge, L. (2000). Selection on age at reproduction in *Drosophila melanogaster*: female mating frequency as a correlated response. *Evolution*, **54**, 2152-2155.

Shakarad, M., Prasad, N. G., Gokhale, K., Gadagkar, V., Rajamani, M., & Joshi, A. (2005). Faster development does not lead to correlated evolution of greater pre-adult competitive ability in *Drosophila melanogaster*. *Biology Letters*, **1**, 91-94.

Sheeba, V., Madhyastha, N. A., & Joshi, A. (1998). Oviposition preference for novel versus normal food resources in laboratory populations of *Drosophila melanogaster*. *Journal of Biosciences*, **23**, 93-100.

Sirot, L. K., Wong, A., Chapman, T., & Wolfner, M. F. (2015). Sexual conflict and seminal fluid proteins: a dynamic landscape of sexual interactions. *Cold Spring Harbor Perspectives in Biology*, **7**, a017533.

Snook, R. R. (2005). Sperm in competition: not playing by the numbers. *Trends in Ecology and Evolution*, **20**, 46-53.

Spieth, H. T. (1974). Courtship behavior in *Drosophila*. *Annual Review of Entomology*, **19**, 385-405.

StatSoft, In. (1995). *STATISTICA for Windows*. StatSoft Inc., Tulsa, OK, USA.

Syed, Z. A., Chatterjee, M., Samant, M. A., & Prasad, N. G. (2017). Reproductive isolation through experimental manipulation of sexually antagonistic co-evolution in *Drosophila melanogaster*. *Scientific Reports*, **7**, 3330.

Teotónio, H., & Rose, M. R. (2000). Variation in the reversibility of evolution. *Nature*, **408**, 463.

Teotónio, H., & Rose, M. R. (2001). Perspective: reverse evolution. *Evolution*, **55**, 653-660.

Teotonio, H., Matos, M., & Rose, M. R. (2002). Reverse evolution of fitness in *Drosophila melanogaster*. *Journal of Evolutionary Biology*, **15,** 608-617.

Trivers, R. (1972). Parental investment and sexual selection. *Sexual Selection & the Descent of Man,* B. Cambell (ed) Aldine de Gruyter, New York, 136-179.

Van Noordwijk, A. J., & de Jong, G. (1986). Acquisition and allocation of resources: their influence on variation in life history tactics. *The American Naturalist*, **128**, 137-142.

Vincent, A., Ahnesjö, I., Berglund, A., & Rosenqvist, G. (1992). Pipefishes and seahorses: are they all sex role reversed? *Trends in Ecology and Evolution*, **7**, 237-241.

Wallace, A. R. (1871). Review of the descent of man and selection in relation to sex, by Charles Darwin. The academy, March 15, 177-183.

Wallace, A. R. (1890). Review of E. B. Poulton's The colours of animals. *Nature* **42,** 289-291.

Wedell, N., Kvarnemo, C., & Tregenza, T. (2006). Sexual conflict and life histories. *Animal Behaviour*, **71**, 999-1011.

Wensing, K. U., Koppik, M., & Fricke, C. (2017). Precopulatory but not postcopulatory male reproductive traits diverge in response to mating system manipulation in *Drosophila melanogaster*. *Ecology and Evolution*, **7**, 10361-10378.

Wigby, S., & Chapman, T. (2004). Female resistance to male harm evolves in response to manipulation of sexual conflict. *Evolution*, **58**, 1028-1037.

Wigby, S., & Chapman, T. (2005). Sex peptide causes mating costs in female *Drosophila melanogaster*. *Current Biology*, **15**, 316-321.

Wigby, S., Sirot, L. K., Linklater, J. R., Buehner, N., Calboli, F. C., Bretman, A., Wolfner, M. F., & Chapman, T. (2009). Seminal fluid protein allocation and male reproductive success. *Current Biology*, **19**, 751-757.

Wigby, S., Perry, J. C., Kim, Y. H., & Sirot, L. K. (2016). Developmental environment mediates male seminal protein investment in *Drosophila melanogaster*. *Functional Ecology*, **30**, 410-419.

Wolfner, M. F. (1997). Tokens of love: functions and regulation of *Drosophila* male accessory gland products. *Insect Biochemistry and Molecular Biology*, **27**, 179-192.

Wolfner M. F. (2009). Battle and ballet: molecular interactions between the sexes in *Drosophila*. *The Journal of Heredity*, **100**, 399-410.

Zikovitz, A. E., & Agrawal, A. F. (2013). The condition dependency of fitness in males and females: the fitness consequences of juvenile diet assessed in environments differing in key adult resources. *Evolution*, **67**, 2849-2860.

Zwaan, B., Bijlsma, R., & Hoekstra, R. F. (1995)-a. Artificial selection for developmental time in *Drosophila melanogaster* in relation to the evolution of aging: direct and correlated responses. *Evolution*, **49**, 635-648.

Zwaan, B., Bijlsma, R., & Hoekstra, R. F. (1995)-b. Direct selection on life span in *Drosophila melanogaster*. *Evolution*, **49**, 649-659.